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Attomolar Ultrasensitive MicroRNA Detection by DNA-Scaffolded Silver-Nanocluster Probe Based on Isothermal Amplification

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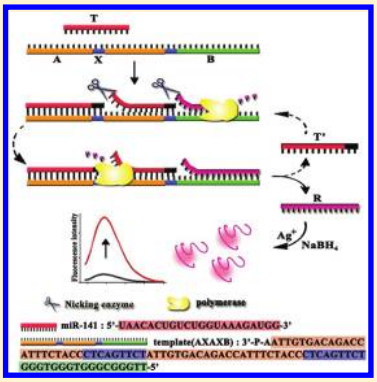
Supporting Information

ABSTRACT: MicroRNAs (miRNAs) play vital roles in a plethora of biological and cellular processes. The levels of miRNAs can be useful biomarkers for cellular events or disease diagnosis, thus the method for sensitive and selective detection of miRNAs is imperative to miRNA discovery, study, and clinical diagnosis. Here we develop a novel method to quantify miRNA expression levels as low as attomolar sensitivity by target-assisted isothermal exponential amplification coupled with fluorescent DNA-scaffolded AgNCs and demonstrated its feasibility in the application of detecting miRNA in real samples. The method reveals superior sensitivity with a detection limit of miRNA of 2 aM synthetic spike-in target miRNA under pure conditions (approximately 15 copies of a miRNA molecule in a volume of 10 μL) and can detect at least a 10 aM spike-in target miRNA in cell lysates. The method also shows the high selectivity for discriminating differences between miRNA family members, thus providing a promising alternative to standard approaches for quantitative detection of miRNA. This simple and cost-effective strategy has a potential of becoming the major tool for simultaneous quantitative analysis of multiple miRNAs (biomarkers) in tissues or cells and supplies valuable information for biomedical research and clinical early diagnosis.



MicroRNAs (miRNAs) are the class of small noncoding ribonucleic acids (RNAs) molecules found in eukaryotic cells with an average of 22 nucleotides. These small RNAs regulate gene expression in plants, animals, and humans by controlling translation. They belong to post-transcriptional regulators that result in translational repression or target degradation and gene silencing via binding to complementary sequences on target mRNA transcripts (mRNAs).[1] It is reported that the human genome may encode over 1500 miRNAs, which may target about 60% of mammalian genes.[2] They are abundant in many human cell types[3] as well as found to regulate multiple genes associated with human cancer, neurological diseases, and viral infections.[4] Especially, the aberrant expression of miRNAs is commonly observed in cancer initiation, oncogenesis, and tumor response to treatments.[5] Detecting miRNAs expression levels in tissues or cells can supply valuable information for biomedical research and clinical early diagnosis.

Northern blotting, quantitative real-time PCR (qRT-PCR), and microarray-based hybridization are the widely used standard methods for analyzing miRNAs. However, these methods have some limitations such as poor reproducibility with interference from cross-hybridization, low selectivity, insufficient sensitivity, time-consuming, or large amounts of sample required. Various new strategies have been developed to improve the detection sensitivity and flexibility, such as RCA-based assay,[6] enzymatic assay,[7] etc. Recently, the exponential isothermal amplification reaction (EXPAR) has found growing interest for detecting DNAs and RNAs with high amplification effciency (10^6~10^9-fold amplification) under a constant temperature within minutes.[8] Usually, SYBR Green I acts as the label in the EXPAR assay and limits the multiplexed assay of miRNA, and SYBR Green I shows both dose-dependent inhibition of amplification reaction and promotion of non-specific amplification.[9] To overcome these drawbacks, Zhang et al. developed a novel miRNA detection method based on the two-stage EXPAR and a quantum dots (QDs)/reporter oligonucleotide/Cy5 complexes nanosensor.[10] This assay is sensitive and specific; however, the QD-based sandwich-type nanosenser needs the Cy5-labeled reporter probes, biotinylated capture probe, and streptavidin-functionalized QDs, inevitably causing complicated or expensive operation. Two template designs greatly increase the experimental complexity and decreases the reaction efficiency. Furthermore, the intrinsic toxicity of QDs due to cadmium release causes risks to human subjects, which limit practical biomedical applications.

Very recently, there has been an explosion of interest in fluorescent silver nanoclusters (AgNCs) synthesis and their application in the area of bioassays.[11] AgNCs with a few atoms, exhibiting size-dependent fluorescence emission, have been developed as a new class of fluorophores, especially the synthesis of fluorescent AgNCs using DNA as scaffolds in aqueous solution has attracted extensive attention.[12] The DNA-

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scaffolded AgNCs (DNA-AgNCs) exhibit outstanding spectral and photophysical properties, and the photoluminescence (PL) emission band can be fine-tuned just by changing the sequence of DNA.[12c] They display excellent photostability, subnanometer size, nontoxicity, biocompatibility, and thus well-suited as a fluorescent probe for biochemical applications. Recent years have witnessed the rapid development of AgNC-based fluorescent probe design and its successful applications in detecting various targets, such as ssDNA, miRNA, and metal ion and for cellular labeling or imaging.[13]

Herein, we present a facile detection of microRNA by target-assisted isothermal exponential amplification (TAIEA) coupled with fluorescent DNA-scaffolded AgNCs. The TAIEA reaction utilizes a unimolecular DNA containing three functional domains as the amplification template, polymerases, and nicking enzymes as mechanical activators and target miRNA as the trigger, which enables the conversion of miRNA to a great amount of the reporter oligonucleotides within minutes. The resultant reporter oligonucleotides can act as scaffolds for the synthesis of fluorescent AgNCs functioning as signal indicators in a label-free and environmental-friendly format. The method demonstrates a detection limit down to 2 aM synthetic spike-in miRNA under pure conditions (in a reaction volume of 10 μL) as well as only 15 copies of miRNA in solution.

EXPERIMENTAL SECTION

Target-Assisted Isothermal Exponential Amplification. The reaction mixtures for target-assisted isothermal exponential amplification (TAIEA) were prepared separately as parts A and B. Part A consisted of 200 nM template, 0.5× Nicking endonuclease buffer (25 mM Tris-HNO_3, pH 7.9 at 25.0 °C, 50 mM $NaNO_3$, 5 mM $Mg(NO_3)_2$, 0.5 mM dithiothreitol), and miRNA. Part B consisted of 0.8 U μL^{-1} RNase inhibitor, 0.4 U μL^{-1} Nt·BstNBI nicking enzyme, 0.05 U μL^{-1} Vent (exo-) DNA polymerase, 1× ThermaoPol buffer (10 mM $NaNO_3$, 20 mM NH_4NO_3, 20 mM Tris-HNO_3, pH 8.8 at 25.0 °C, 2 mM $Mg(NO_3)_2$, 0.1% Triton X-100), 250 μM deoxynucleotide triphosphates (dNTPs), and DEPC-treated water. Part A was prepared and incubated at 88 °C for 10 min to denature the template and miRNA and then cooled to room temperature. After that, parts A and B were mixed and the TAIEA reaction was performed in a volume of 10 μL at 53 °C for 50 min and stored at 4 °C for further use.

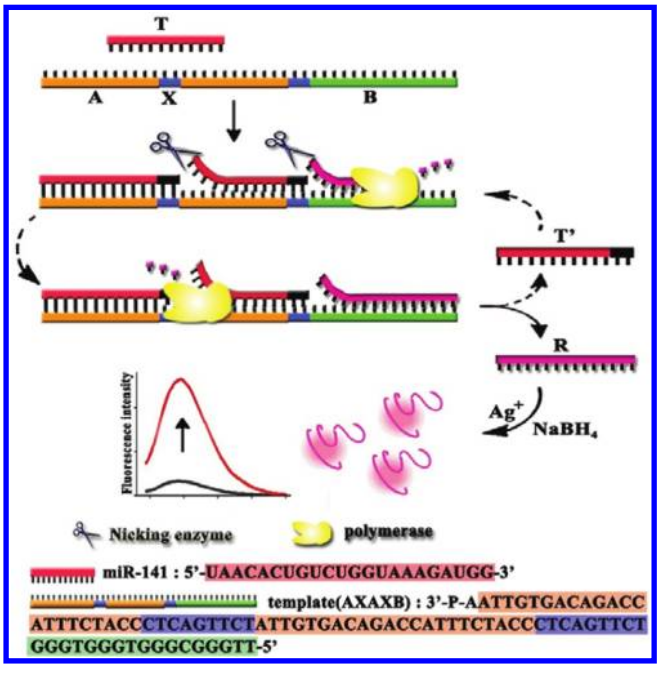
Preparation of DNA-Scaffolded Silver Nanoclusters and Fluorescence Measurements. Briefly, the resultant products of TAIEA reaction was mixed with 497.5 μM $AgNO_3$ in the sodium citrate buffer (10 mM, pH 7.0) and centrifuged at 12 000 rpm for 5 min at room temperature to collect the supernatant. The supernatant was incubated at room temperature, in the dark, for 15 min. Then, 18 μM freshly prepared $NaBH_4$ was added and the reaction mixture was incubated at room temperature, in the dark, for 1 h. Following reduction of Ag^+ ions by $NaBH_4$, the as-prepared fluorescent DNA-AgNCs were produced with fluorescence emission at 644 nm upon the excitation at 574 nm. The control samples of DNA-AgNCs without TAIEA reaction were prepared using the aforementioned methods with minor modification, that is, various concentrations of **Cluster** (CCCACCCACCCGCCCAA), 497.5 μM $AgNO_3$, and the same component of TAIEA reaction except for miRNA were sequentially added and mixed with sodium citrate buffer (10 mM, pH 7.0), and centrifuged at 12 000 rpm for 5 min at room temperature to collect the supernatant. The supernatant was incubated at room temperature, in the dark, for 15 min. With that, 18 μM $NaBH_4$ was added and the reaction mixture was incubated at room temperature, in the dark, for 1 h. Following reduction of Ag^+ ions by $NaBH_4$, fluorescent DNA-scaffolded silver nanoclusters (DNA-AgNCs) were produced with fluorescence emission at 644 nm with the excitation at 574 nm.

Preparation of Cellular Extracts. Human pancreatic cancer cell lines (AsPc-1), human prostate carcinoma cell lines (22Rv1), human hepatocellular carcinoma cell lines (BEL-7404), human cervical cancer cell lines (HeLa), and human breast cancer cell lines (MDA-MB231) were obtained from the cell bank of type culture collection of the Chinese Academy of Sciences (Shanghai, China). AsPc-1 and 22Rv1 cells were, respectively, cultured in RPMI Medium 1640, supplemented with 10% fetal bovine serum (FBS) and 100 U mL^{-1} penicillin-streptomycin at 37 °C in a humidified 5% CO_2 incubator. HeLa and BEL-7404 cells were, respectively, cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 U mL^{-1} penicillin-streptomycin at 37 °C in a humidified 5% CO_2 incubator. The cells were grown with fresh medium at 50 mm glass-bottom dishes. Then the cells were harvested by trypsinization and washed with fresh medium (three times) and suspended in fresh medium for the following studies. The preparation of cellular extracts was conducted according to the reported method with minor modifications.[14]

RESULTS AND DISCUSSION

Strategy of miRNA Assay. As shown in Scheme 1, the unimolecular template involves five regions (**AXAXB**). Two repeat sequences of **A** are complementary to the target miRNA. Also, two repeat sequences of **X** represent the "heart" of the template, upon their replication; the complementary strand

Scheme 1. Detection of miRNA with Attomolar Sensitivity Based on Target-Assisted Isothermal Exponential Amplification (TAIEA) Coupled with Fluorescent DNA-Scaffolded AgNC Probe



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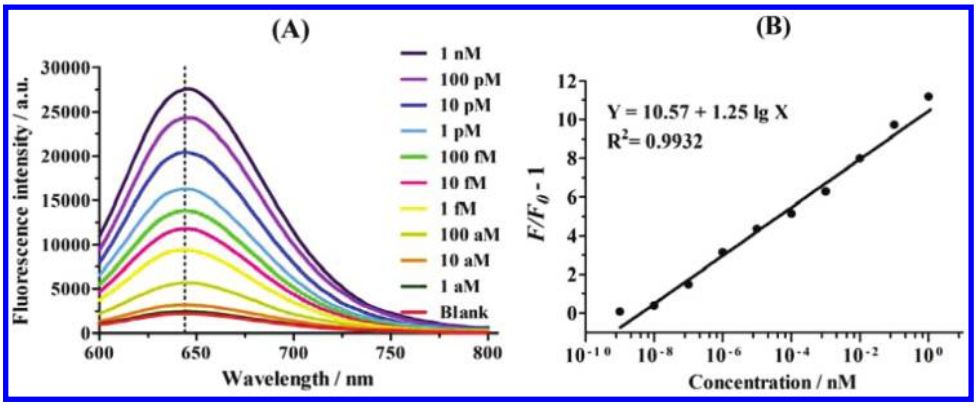

Figure 1. (A) Emission spectra representing the dose−response fluorescence DNA-AgNCs from the different concentrations of miR-141 (0, 1 aM, 10 aM, 100 aM, 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, and 1 nM); (B) plot of fluorescence ratio $(F/F_0 - 1)$ of the resultant fluorescent DNA-AgNCs at 644 nm with the different concentrations of miR-141, where F_0 and F are the fluorescence intensities in the absence and the presence of miR-141 inputs, respectively.

includes the specific sequence for nicking by Nt·BstNBI. The sequence of **B** is complementary to the reporter oligonucleotide **R** acting as a scaffold for the synthesis of fluorescent AgNCs. The TAIEA reaction was initiated through the hybridization of the **A** with the target miRNA **T** (as a trigger). The formation of a partial duplex can be extended in the presence of polymerase/dNTPs, resulting in a stable double-stranded DNA duplex with two recognition sites **X** for the nicking enzyme Nt·BstNBI. The cleavage of the first recognition site results in the initiation of a secondary polymerization cycle while displacing the sequence **T′** complementary to **A**, leading in an exponential amplification of the trigger. The sequence of **T′** was the same as that of the target miRNA **T** except for the change of U to T and the change of ribonucleotides to deoxyribonucleotides. In addition, the cleavage of the second recognition site can produce the reporter oligonucleotide **R** through a polymerization and displacement reaction cycle. The production of reporter oligonucleotide **R** was verified by nondenaturating PAGE. As shown in Figure S1 in the Supporting Information, a characteristic band of reporter oligonucleotide **R** (17 nt) was observed in the presence of miR-141; in contrast, the negative control without miR-141 or the competing stimulus including miR-429, miR-21, miR-200b, and let-7d showed negligible bands. The level of reporter oligonucleotide **R** yield was 89% calculated on the basis of the equation: $(R - N)/R$, where R is the band intensity value of reporter oligonucleotide **R** in the presence of miRNA and N is the band intensity value of the negative control without miRNA.

After amplification, the reporter oligonucleotide **R** was acting as a scaffold for the synthesis of fluorescent silver nanoclusters in the presence of Ag^+ through the reduction of $NaBH_4$. The DNA-AgNCs scaffolded with the reporter oligonucleotide displayed fluorescence emission at 644 nm upon the excitation at 574 nm (Figure S2 in the Supporting Information). To investigate the feasibility of quantitative detection, a series of synthesized reporter oligonucleotide (**Cluster**, 5′-CCCACC-CACCCGCCCAA-3′) with known concentrations were employed as the scaffolds. The results showed that the fluorescence is sensitive to the concentration of reporter sequence, and a linear equation can be obtained from the concentration range of 0−1 μM ($Y = 9354X + 14.3$, $R^2 = 0.998$), where Y is the fluorescence intensity and X is the concentration. The limit of detection of reporter oligonucleotide based on 3σ was approximately 5 nM (Figure S3 in the Supporting Information).

Sensitivity of miRNA Assay. We first optimized the amplification and sensing conditions, including the amount of template, amplification reaction temperature, and the concentration of Ag^+ for the synthesis of fluorescent DNA-AgNCs (see the Supporting Information). Unless noted otherwise, all experiments were carried out under the optimal conditions: 200 nM template, 0.4 U μL^{-1} Nt·BstNBI nicking enzyme, 0.05 U μL^{-1} Vent (exo-) DNA polymerase for TAIEA amplification in a reaction volume of 10 μL at 53 °C and 497.5 μM Ag^+ for the synthesis of DNA-AgNCs.

To test the viability of our design strategy, miR-141 (a miRNA up-expressed in prostate cancer) was selected and artificially synthesized as a model. The different concentrations (0, 1 aM, 10 aM, 100 aM, 1 fM, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, and 1 nM) of miR-141 from one stock solution were added to the TAIEA solution, and the resultant products of TAIEA reaction was then as the scaffold for the synthesis of fluorescent AgNCs following reduction of Ag^+ ions by $NaBH_4$.

Figure 1A demonstrates the emission spectra of the resultant DNA-AgNCs from the different concentrations of miR-141. As expected, a gradual increase in the fluorescent peak at 644 nm was clearly observed with the increasing concentration of miR-141 from 0 to 1 nM. From Figure 1B, it can be seen that the fluorescence ratio $(F_0/F - 1)$ is sensitive to the concentration of miR-141, the fitting range is from 1 aM to 1 nM with a logarithmic equation $Y = 10.6 + 1.25 \lg X$, where Y is the fluorescence ratio $(F_0/F - 1)$ and X is the concentration of miR-141 (regression coefficient $R^2 = 0.993$). The limit of detection of miR-141 based on 3σ was approximately 2 aM synthetic spike-in miRNA under pure conditions. Also for real application, the limit of detection of miR-141 in cell lysates can be obtained to be 10 aM by adding a spike-in target miRNA to cell extracts. To the best of our knowledge, it is one of the most sensitive methods for detection and analysis of miRNA. The detection sensitivity of the current assay method can be significantly improved by 7−8 orders of magnitude over the previously reported "turn off" AgNC-based assay for miRNA[13b] and better by at least 5 orders of magnitude compared to the single-molecule detection with two organic fluorophores as the labels.[15] In this study, the miR-141 solutions with different concentrations were also tested using the qRT-PCR kit (Perfect Real Time, TaKaRa Ltd.) according to the manufacturer's

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instructions, and the limit of detection of the kit-based method was about 100 fM (Figure S6 in the Supporting Information). That is, our proposed method can improve the sensitivity as much as 4 orders of magnitude over the commercial kit-based method. The substantial improvement of the TAIEA method in the sensitivity is mainly attributed to two distinguishing features: (1) the exponential amplification leads to the effective generation of more than 10^9 reporter molecules from one miRNA target by designing a unimolecular template for TAIEA reaction; (2) the near-zero background noise of the "turn on" single-AgNC-based nanoprobe with high sensitivity. A series of eight repetitive measurements with 1 fM miR-141 was used for investigating the precision of the proposed method and obtained a relative standard deviation (RSD) of 5.4%, demonstrating an excellent reproducibility of the assay.

The proposed ultrasensitive method might be well qualified for detection of low-abundance miRNA, which acts as promising markers for cancers, in early clinical diagnosis. However, some pathologies such as cancers are typically associated with abnormal expression of several miRNAs. The use of miRNA for clinical diagnostics requires accurate simultaneous quantitative analysis of multiple miRNAs. Richards et al. reported that the photoluminescence (PL) emission band of DNA-AgNCs can be fine-tuned just by changing the sequence of used DNA.[12c] Enlightened with this fact, we investigated four types of fluorescent DNA-AgNCs using various reporter scaffolds including **Cluster**, **C1** (5′-CCCTTAATCCCC-3′), **C2** (5′-CCCTTACTCCCC-3′), and **C3** (5′-CCCTAACTCCCC-3′). It was observed that the PL emission band of these four DNA-AgNCs can be differentiable (Figure S7 in the Supporting Information), which are well suited for optical encoding and multiplexing applications. The preliminary results indicated that the proposed method has great potentials for multiplexed detection of several miRNAs.

Specificity of miRNA Assay. It was a great challenge to discriminate differences between miRNA family members due to their high similarity. The miR-141 belongs to the miR-200 family, which contains five members of miR-200a, miR-200b, miR-200c, miR-141, and miR-429. Three members of the miR-200 family (miR-141, miR-200b, and miR-429), miR-21, and let-7d (Table S1 in the Supporting Information) were used for the evaluation of sequence-specificity of the AgNC-based TAIEA assay. As shown in Table S1 in the Supporting Information, miR-141 shows high similarity with miR-200b and miR-429. The miR-21 is highly overexpressed in breast tumors, and let-7d is conservative among human cells. Five artificially synthesized target miRNAs were analyzed at the same concentration of 10 fM, 100 fM, and 1 pM, respectively. The results were shown in Figure 2. The signal $F/F_0 - 1$ from 10 fM miR-141 was approximately 4-fold higher than that of 1 pM miR-200b and miR-429 (target miRNA is present in a 100-fold lower concentration compared to nontarget related miRNAs). Moreover, there was nearly negligible fluorescent change in the presence of miR-21 and let-7d. Thus, the specificity in our work can be well addressed when the target miRNA is present in low concentrations compared to nontarget miRNAs. These results demonstrate that the specificities of the presented method are high enough to discriminate between the three miR-200 family members and show the significant advantage of high selectivity in comparison with the previous methods for discriminating differences between miRNA family members and other stimulus.[15]

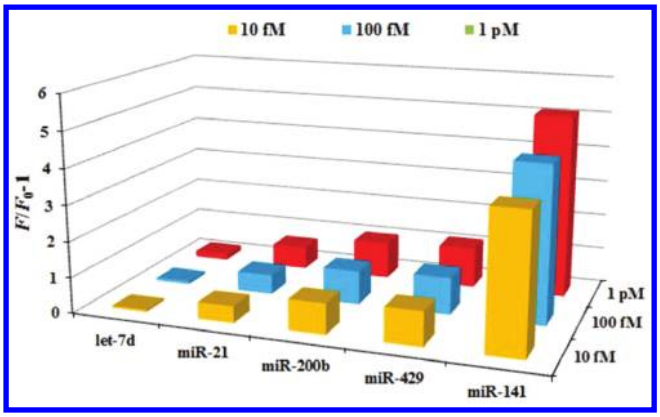

Figure 2. Specificity of miRNA assay. Bars representing the fluorescence ratio ($F/F_0 - 1$) of the fluorescent DNA-AgNCs from the different inputs of miR-141, miR-200b, miR-429, miR-21, and let-7d with the same concentration of 10 fM, 100 fM, and 1 pM, respectively, where F_0 and F are the fluorescence intensities at 644 nm in the absence and the presence of inputs indicated, respectively.

Assays for miRNA in Cell Lysate. To determine whether this method could be applied to target miRNA detection in real samples, we first prepared a 22Rv1 cell lysate sample, which is known to up-regulate miR-141 and four other cell lysate samples (BEL-7404, AsPc-1, HeLa, and MDA-MB231). The concentrations of miR-141 in five different cell lysate samples were measured by the qRT-PCR and the proposed method, respectively. The experimental data were listed in Figure 3,

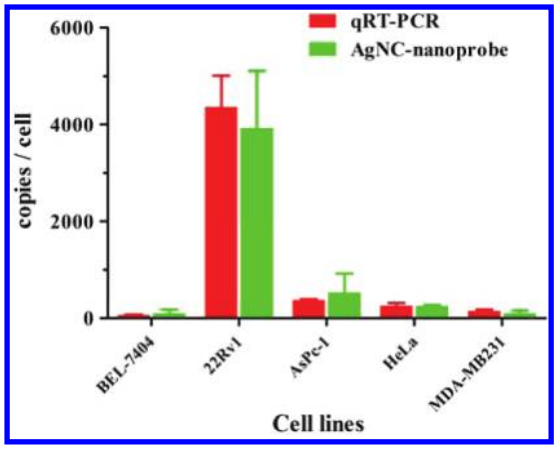

Figure 3. Cancer-derived miRNAs are detectable in cell lysates. Bars represent the copies of miR-141 in the different cancer cells including BEL-7404, 22Rv1, AsPc-1, HeLa, and MDA-MB231 detected with the methods of qRT-PCR (red bars) and the presented method (green bars), respectively.

which demonstrated that the cell lysate extracted from 22Rv1 cell lines has higher concentration of miR-141 more than that of other cell lines, and each 22Rv1 cell contains ~4500 copies of miR-141. The results were consistent with a previous report that the levels of miR-141 can distinguish patients with prostate cancer from healthy controls or other cancer controls.[16] Moreover, various concentrations of miR-141, which were spiked into the MDA-MB231 cell lysates, were also effectively detected (Table S2 in the Supporting Information). The method reveals the good recovery rates of standard addition from 98.7 to 102.1%. These results indicated that TAIEA coupled with fluorescent DNA-scaffolded AgNCs has a promise

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in practical application with great accuracy and reliability for miRNA detection.

CONCLUSIONS

In this work, we have developed a novel method to quantify miRNA expression levels as low as attomolar sensitivity by target-assisted isothermal exponential amplification (TAIEA) coupled with fluorescent DNA-scaffolded AgNCs and demonstrated its feasibility in the application of detection of miRNA in real samples. The TAIEA reaction using the unimolecular DNA template enables the effective conversion of miRNA to a great amount of the reporter oligonucleotides (10^9-fold) within minutes. The resultant reporter oligonucleotides can act as scaffolds for the synthesis of fluorescent AgNCs functioning as signal indicators. The novel method demonstrates superior ultrasensitivity with a detection limit of miRNA of 2 aM synthetic spike-in miRNA under pure conditions (approximately 15 copies of a miRNA molecule in a volume of 10 μL). To the best of our knowledge, it is one of the most sensitive methods for detection and analysis of miRNA. The method also shows high selectivity for discriminating differences between miRNA family members. Moreover, the concept can easily be extended to construct a series of probes by simply changing the functional domain sequence **A** (see Scheme 1), corresponding to specific miRNA in the unimolecular DNA template, for detecting the different kind of miRNAs. More importantly, the photoluminescence emission band of DNA-AgNCs can be fine-tuned just by using the oligonucleotide sequence. Multiplex detection of miRNAs can be potentially realized by designing multiple templates containing different functional domain sequences **B** (generating the fluorescent DNA-AgNCs with different emission peaks). The experimental results demonstrated that this simple and cost-effective method has a potential of becoming the major tool for simultaneous ultrasensitive quantitative analysis of multiple miRNAs (biomarkers) in tissues or cells and supply valuable information for biomedical research and clinical early diagnosis.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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