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An "Off–On" Electrochemiluminescent Biosensor Based on DNAzyme-Assisted Target Recycling and Rolling Circle Amplifications for Ultrasensitive Detection of microRNA

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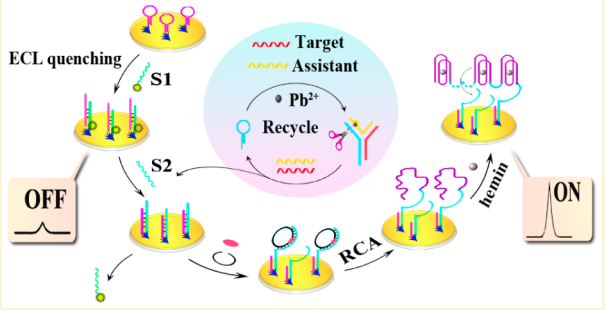
Supporting Information

ABSTRACT: In this study, an off–on switching of a dual amplified electrochemiluminescence (ECL) biosensor based on Pb^{2+}-induced DNAzyme-assisted target recycling and rolling circle amplification (RCA) was constructed for microRNA (miRNA) detection. First, the primer probe with assistant probe and miRNA formed Y junction which was cleaved with the addition of Pb^{2+} to release miRNA. Subsequently, the released miRNA could initiate the next recycling process, leading to the generation of numerous intermediate DNA sequences (S2). Afterward, bare glassy carbon electrode (GCE) was immersed into $HAuCl_4$ solution to electrodeposit a Au nanoparticle layer (depAu), followed by the assembly of a hairpin probe (HP). Then, dopamine (DA)-modified DNA sequence (S1) was employed to hybridize with HP, which switching off the sensing system. This is the first work that employs DA to quench luminol ECL signal, possessing the biosensor ultralow background signal. Afterward, S2 produced by the target recycling process was loaded onto the prepared electrode to displace S1 and served as an initiator for RCA. With rational design, numerous repeated DNA sequences coupling with hemin to form hemin/G-quadruplex were generated, which could exhibit strongly catalytic toward H_2O_2, thus amplified the ECL signal and switched the ON state of the sensing system. The liner range for miRNA detection was from 1.0 fM to 100 pM with a low detection limit down to 0.3 fM. Moreover, with the high sensitivity and specificity induced by the dual signal amplification, the proposed miRNA biosensor holds great potential for analysis of other interesting tumor markers.



MicroRNA (miRNA) are a fresh emerging series of endogenetic, small, noncoding single-stranded RNAs,[1,2] primarily functioning as significant regulators to regulate fundamental cellular procedures via the modulation toward the expression of target genes.[3−6] Until now, various analytical methods, including electrochemical,[7] fluorescent,[8] chemiluminescent,[9] electrochemiluminescence (ECL),[10] and surface plasmon resonance,[11] have been implemented to realize sensitive detection of miRNA. Among these protocols, ECL, because of its rapidness, high detection sensitivity, simplified operation, and low cost, has attracted particular attention and offered new opportunities for applications in biological analysis.

In ECL system, the target recycling amplification techniques related to nicking-nuclease have been regarded as the most frequently used methods, such as nicking endonuclease signal amplification[12−14] and exonuclease III-aided signal amplification.[15−17] Nevertheless, these amplified strategies suffered from negative factors, involving high cost and possible false responses, because of the inactivation of the enzyme. Lead-requiring DNAzyme is a sequence-specific nuclease that can catalyze a wide succession of biological and biochemical reactions, which could smooth over the above disadvantages. Lead-requiring DNAzyme has received remarkable interests, because of its merits of high sensitivity, easy operation, low cost to produce, and ability to be renatured many times without losing activity.[18] Such a lead-requiring DNAzyme compound have been isolated in test tubes[19,20] and is highly affinitive for specific metal ions, including Pb(II),[21−23] Cu(II),[24] and Zn(II).[25,26] A typical Pb^{2+}-requiring DNAzyme design called "8-17" is composed of a substrate strand (17DS) and an enzyme strand (17E).[21,22] In the presence of Pb^{2+}, the specific cleavage of substrate strand was nicked, resulting in the substrate strand changing into two fragments. To the best of our knowledge, there is little research that focuses on adapting a Pb^{2+}-lead dependent DNAzyme in an ECL biosensor for miRNA detection.

As one of the most extensively implicated luminophores in ECL, luminol has some outstanding properties, including nontoxicity, low oxidation, moderate price, and high light-emitting quantum yield.[27,28] The luminol ECL response could be increased by the presence of H_2O_2, since the decomposition of H_2O_2 may generate a large number of reactive oxygen

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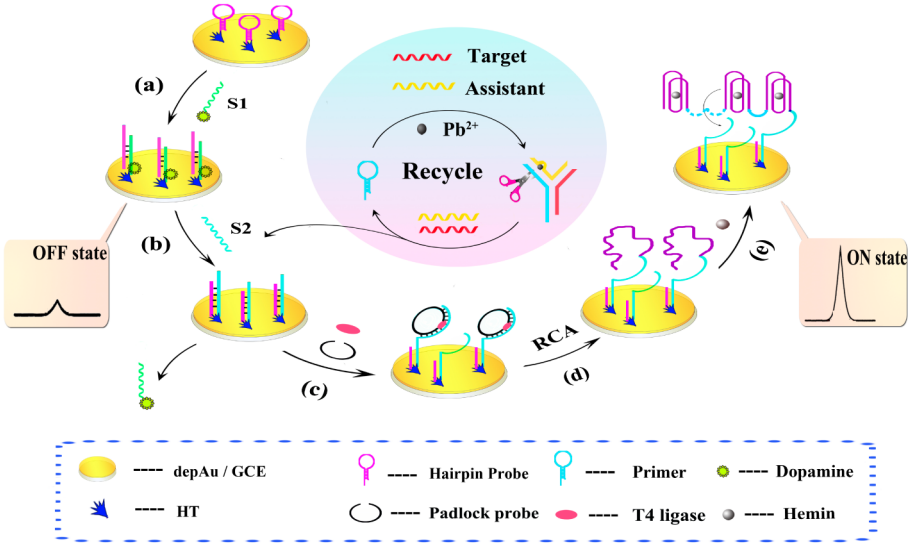
Scheme 1. Schematic Diagram of Dual Amplified Assay Conducted Procedure: (a) Modification of S1, (b) Displacement of S1 by S2, (c) Incubation of T4 Ligase and Padlock Probe, (d) RCA Process, and (e) the Formation of Hemin/G-quadruplex



species (ROSs), which could further oxidize luminol for the ECL signal.[29,30] On the side of an HRP-mimicking DNAzyme, Hemin/G-quadruplex showed strong catalytic ability toward H_2O_2 in order to enhance the luminol ECL signal.[31,32] In addition, the quenching of luminol ECL signal was also studied.[33−36] However, no study concerning the luminol ECL signal being quenched by dopamine (DA) has not been reported until now. Importantly, the quenching effect by DA is much more significant, which may be attributed to the fact that the oxidization of DA consumes a notable amount of ROSs, resulting in the remarkable decrease of the ECL signal of luminol. Most of the previous research has focused only on "signal-on"[37] or "signal-off"[38] ECL biosensors, leading to false positive ECL signals rooting in disturbing species, which restrict the employment and practice in analysis of the real samples. The skillful integration of the off−on mode endows the biosensor with preferable abilities, including low background signal, high sensitivity, specific recognition and sophistication, paving the way for further improvement of the detection sensitivity. To the best of our knowledge, little attentions has been paid to designing an off−on mode biosensor for miRNA detection in a luminol ECL system.

Herein, as shown in Scheme 1, by coupling Pb^{2+}-requiring DNAzyme-assisted target recycling amplification with Y junction, we developed a dual amplified ECL biosensor for highly sensitive miRNA detection in an off−on manner for the first time. The primer probe was first incubated with the assistant probe and the target RNA to form the Y junction structure. Participation of Pb^{2+} cleaved the Y junction on the cleavage site, bringing about the release of the target and the generation of intermediate DNA (S2). Meanwhile, the released target RNA again hybridized with other primer probes and assistant probes to initiate another recycling and generate abundant S2. Subsequently, the treated glassy carbon electrode (GCE) was electrodeposited with $HAuCl_4$, which provided an outstanding Au nanoparticles platform to modify numerous HP through Au−S bonding. Next, hexanethiol (HT) was employed to block the nonspecific binding sites. Furthermore, S1 was introduced onto the prepared GCE to unfold the hairpin loop of HP DNA and hybridize with it. Thus, the ECL signal was reduced due to the quenching effect of DA toward luminol, which indicated that the system presented the OFF state. S2 then was dropped on the prepared electrode to displace S1. The end of S2 further acted as a primer to initiate the rolling circle amplification (RCA) reaction, which could increase the signal via the formation of massive repeated sequences with specificity and high efficiency.[36,37] The generated multiplication of long DNA sequences associated with hemin to form hemin/G-quadruplex complexes, leading to the sensing system being switched on, as indicated by a strong ECL signal. In general, combining Pb^{2+}-requiring DNAzyme-assisted target recycling amplification together with RCA, an ECL biosensor on the basis of an off-on switching mode for ultrasensitive miRNA detection has been successfully developed. The proposed strategy offered a versatile tool and a clinical biomedicine technique for the signal amplification of ECL biosensing.

EXPERIMENTAL SECTION

Reagents and Materials. Luminol (98%), $HAuCl_4{\cdot}4H_2O$, $K_3Fe(CN)_6$, hemin, and hexanethiol (96%, HT) were purchased from Sigma−Aldrich (USA). T4 DNA ligase and Phi29 DNA polymerase were purchased from Fermentas (Lithuania). Lead nitrate (Pb^{2+}) and hydrogen peroxide solution (30%) were obtained from Chemical Reagent (Chongqing, China). Phosphate-buffered solution (PBS) (pH 7.4) was employed, as in the previous work.[32] Buffered to dilute miRNA-155 and DNA chains were produced, according the previous work.[38]

All the DNA oligonucleotides of the proposed work were custom-synthesized by TaKaRa (Dalian, China), and were maintained at −20 °C for further use. The cell lysates were purchased from Well-Biology Co. (Changsha, China). The sequence information is shown as follows:

Hairpin probe:

5′-GATGTAAATTACCTTACATCTCTTCC-(CH_2)-HS-3′

S1:

5′-COOH-GGAAGAG-3′

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Primer probe:

5′-AATTACGATTAGACTCACTATArGGAAGAGATGTAA
-GGTAATAGTGAGTCTAATCGT-3′

Assistant probe:

5′-CATCTCTTCTCCGAGCCGGTCG
-AAATAGTGAGTCACTATCCCCA-3′

MiRNA:

5′-UUAAUGCUAAUCGUGAUAGGGGU-3′

Padlock probe:

5′-p-**ACTCACTATTA**AAAACCCAACCCGCCCTACCC

In the latter expression, the bold portion matches a part of S2; the underlined letters are completely complementary with the G-quadruplex sequence, and the "p" at the 5′ end indicates phosphate).

Instrumentation. Electrochemical (EC) analysis was conducted on a Model CHI 660A electrochemistry workstation (Shanghai CH Instruments, China). ECL detection system was carried out using a Model MPI-E electrochemiluminescence analyzer (Xi'an Remax Electronic Science & Technology Co. Ltd., Xi'an, China) with the photomultiplier tube set at 800 V and the potential scan was from −0.1 V to 0.4 V. Both the EC and ECL systems were equipped with a three-electrode system including GCE (Φ = 4 mm) working electrode, a platinum wire counter electrode, and an Ag/AgCl (3 M KCl) reference electrode. Quantitative real-time PCR analysis was conducted in Well-Biology Co. (Changsha, China). The pH detection was obtained with a pHS-3C meter (MP 230, Mettler−Toledo, Switzerland). Polyacrylamide gel electrophoresis (PAGE) was carried out using a Model DYY-8C electrophoretic device (Beijing WoDeLife Sciences Instrument Company, Ltd.).

Preparation of the S1 Composite, Y-Junction, and Pb^{2+}-Induced DNAzyme-Assisted Target Recycling. The S1 composite was obtained as follows. First, 2 μM S1 was added into 0.5 mL of a mixture of EDC and NHS (4:1) and stirred over 12 h in order to activate the carboxyl of S1. Subsequently, 500 μL of 100 μM DA solution was added in the planned S1 mixture, stirring for 4 h simultaneously to obtain the S1 composite. The formation of a Y-junction and Pb^{2+}-induced DNAzyme-assisted target recycling were prepared according the method reported in Plaxco's group.[21]

Switchable Off−On Biosensor Based on RCA Generation of Hemin/G-quadruplex Complexes. Scheme 1 illustrated the detection procedure of the proposed biosensor. Before modification, the glassy carbon electrode (GCE) was polished to a mirror with 0.3 and 0.05 μm alumina slurry, followed by sonication with distilled water. After being dried in air, the electrode was electrodeposited in $HAuCl_4$ solution at −0.2 V for 30 s to gain a Au nanoparticle layer. Then, 10 μL of 2.0 μM HP DNA was attached onto the Au nanoparticle layer overnight. After blocking with 1.0 mM HT for 40 min, 20 μL of S1 composite was modified on the biosensor. The resulting biosensor was incubated with 20 μL of the S2 (intermediate DNA sequences) for 2 h. For subsequent RCA process, 10 μL of a solution containing 1× ligase buffer, 0.2 U T4 ligase, and 50 nM padlock probe was deposited dropwise on the resulting electrode to hybridize with the S2 and reacted for 1 h at room temperature. Then, a circular template was achieved through the use of T4 ligase, which linked the 5′ and 3′ end of the padlock probe. The needless templates were eliminated by washing twice with doubly distilled water. After that, RCA procedure was triggered by the injection of dNTP (1.0 mM) and phi29 DNA polymerase (0.5 U) was diluted according the operation attached. The RCA reaction was preceded for 1 h at 37 °C. All the hairpin DNA chains were heated to 95 °C for 2 min to open the loop. Finally, 0.2 mM hemin was applied on the proposed electrode for 30 min, to generate the hemin/G-quarduplex structure. A 1.0×10^{-2} M stock solution of luminol was prepared by dissolving it in 0.1 M NaOH. The designed biosensor was detected in 2 mL PBS (pH 7.4) containing 1.0×10^{-4} M luminol and 0.35 M H_2O_2 to evaluate the ECL response.

RESULTS AND ANALYSIS

Electrochemical and PAGE Characterization of the Biosensor. In order to follow the step-by-step construction of the biosensor, the stepwise procedure used is shown in Figure 1. A pair of well-defined redox peaks were achieved when bare

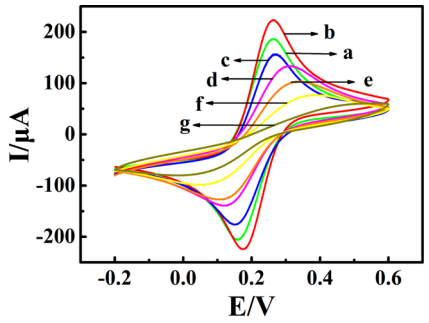

Figure 1. Electrochemical characterization of the biosensor: (a) bare glassy carbon electrode (GCE), (b) depAu/GCE, (c) HP modified depAu/GCE, (d) blocking with HT, (e) S1/HT/HP/depAu/GCE, (f) S2 replacing S1, and (g) introducing of RCA. The CVs of the biosensor were carried out from −0.2 V to 0.6 V in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution.

GCE was detected (curve a). There was a significant enhancement when the electrode was modified with Au nanoparticles (curve b), because Au nanoparticles as electroconductive material could accelerate the electron transfer. After the introduction of HP, a remarkable decline was achieved ascribe to that HP can impede the electron transfer (curve c). After blocking with HT, the CV response remained declined (curve d). The CV signal decreased notably when the biosensor was incubated with S1, because of their nonelectroactive character (curve e). Subsequently, the CV response continued to decrease when S2 was introduced on the surface of the electrode, testifying that S2 inhibited the transmission of electrons (curve f). As expected, the current response was greatly reduced after the electrode was employed in a subsequent RCA reaction, because there was repulsive effect resulting from the long DNA sequences. Besides, PAGE was also carried out to demonstrate the mechanism of the proposed methods; the specific details are shown in the Supporting Information (Figure S4).

ECL Performance of the Biosensor. The ECL signal was monitored gradually to record the behavior of the electrode modification. As can be seen in Figure 2, a very low ECL signal was achieved from the bare GCE (curve a). The ECL signal then was enhanced after depAu modification, since Au nanoparticles can facilitate the electron transfer. However, given the assembly of HP probe, the ECL intensity decreased

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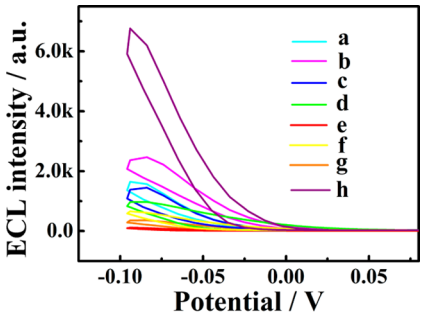

Figure 2. Cathodic ECL response for each immobilized step: (a) bare GCE, (b) depAu/GCE, (c) HP immobilized on depAu/GCE, (d) HT blocking, (e) incubated with S1, (f) S2 replacing S1, (g) RCA procedure, and (h) hemin modification. The biosensor was analyzed in 2 mL PBS (pH 7.4) contained 1.0×10^{-4} M luminol and 3.5×10^{-5} M H_2O_2.

remarkably (curve c). When the electrode was blocked with HT, the ECL response declined continually (curve d). We then noticed that there was almost no ECL signal that resulted on the addition of S1 composite, suggesting that DA which was attached on S1 quenched the luminol ECL intensity (curve e). It is significant to mention that the superiority of using an off−on system over the conventional one achieved an ultralow background ECL signal, further increased the sensitivity, and included a low detection limit and a wider range of detection. Nevertheless, the ECL response was enhanced after S2 replaced quencher S1 (curve f). The signal was further declined after the RCA procedure, which was basically due to the suppression of the formation of the G-quadruplex sequences (curve g). Finally, when the electrode was incubated with hemin, a significantly amplified ECL signal was observed (curve h). Such ECL response can be assigned to that the formation of hemin/G-quadruplex catalyzed H_2O_2 turning into ROSs and further enhancing the ECL signal. Furthermore, a comparison of the target recycling amplification with both target recycling and RCA amplification was employed to highlight the performance of two-step amplification (see Figure S6 in the Supporting Information).

Optimal Conditions for the ECL Biosensor. To achieve optimal sensing performance, experimental parameters including the concentrations of Pb^{2+} and hemin were investigated. Figure 3A displayed the ECL behavior of the biosensor in known concentrations of Pb^{2+}. As anticipated, the ECL response was enhanced rapidly with the augmentation of Pb^{2+}, and the maximum ECL response was achieved at 10 μM; the addition of increasing concentrations of Pb^{2+} resulted in more cleaved Y-junctions and S2. As a result, an optimal Pb^{2+} concentration of 10 μM was used in the following experiments.

The concentration of hemin also had significant influence on the biosensor, because of its strong catalytic ability toward H_2O_2. As can be seen in Figure 3B, ECL intensity increased gradually when there was more hemin modified, demonstrating the formation of hemin/G-quadruplex resulted in significantly enhanced of ECL intensity. Finally, it stabilized when the concentration of hemin was 0.4 mM. Thus, 0.4 mM was the optimal choice of hemin in the proposed biosensor. In addition, the optimal incubation time of RCA reaction was 60 min (see Figure S1 in the Supporting Information).

ECL Analysis of the miRNA-155 Biosensor. The quantitative measurements of the obtained ECL biosensor were examined under the off−on mode by adding known concentrations of miRNA under the optimal conditions. From the results shown in Figure 4, the elevated concentration of the miRNA from 1.0 fM to 100 pM caused a gentle increase in the ECL signal. The detection limit of miRNA was 0.3 fM. The linear equation was

$$I = 3318.6 \log c + 10267.9$$

with the correlation coefficient of $R = 0.9988$, where I represents the ECL response and c represents the concentration of the miRNA. Reading the responses, we can determine that the as-prepared biosensor exhibited high sensitivity with a much lower detection limit and determine miRNA quantitatively. Besides, the comparison of the performances of the proposed biosensors with other biosensors and other methods were discussed, and the results are listed in the Supporting Information (Tables S1 and S2), demonstrating that our work

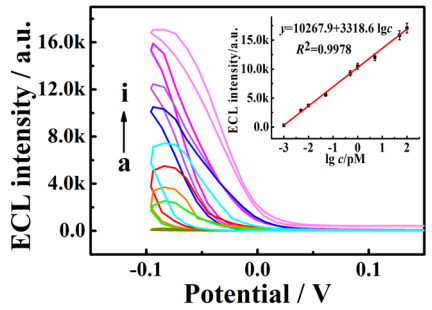

Figure 4. Cathodic ECL response of the designed biosensor in the presence of different concentrations of miRNA-155): (a) 0.001, (b) 0.005, (c) 0.01, (d) 0.05, (e) 0.1, (f) 1.0, (g) 5.0, (h) 50, and (i) 100 pM. Inset shows a calibration plot of the miRNA analysis.

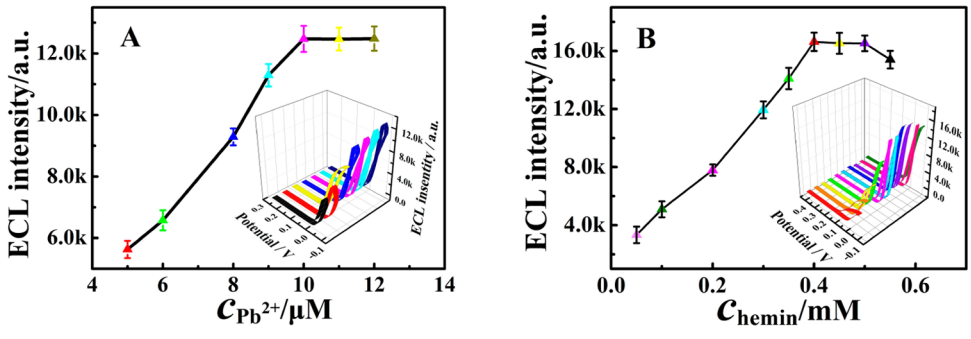

Figure 3. Cathodic ECL intensity is dependent on the concentration of (A) Pb^{2+} and (B) hemin of the designed biosensor.

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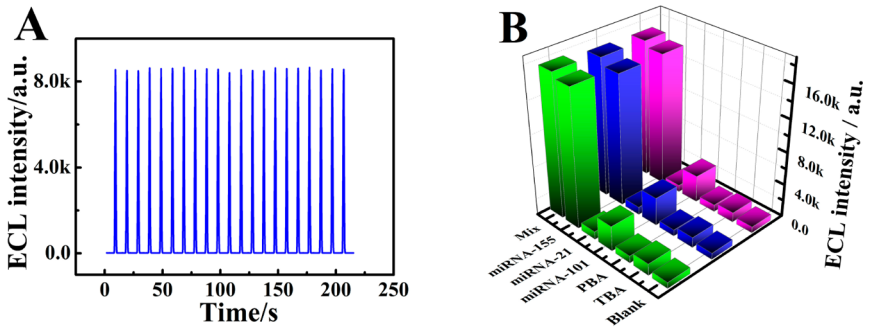

Figure 5. (A) Stability of the as-proposed biosensor when the concentration of miRNA-155 was 0.5 pM. (B) Specificity of the biosensor when employed with a blank test, 500 pM of TBA, 500 pM of PBA, 500 pM of miRNA-101, 500 pM of miRNA-21, 50 pM of miRNA-155, and 50 pM of miRNA-155 when all the above coexisted.

exhibited a low detection limit. In addition to this, we explored the designed biosensor without DA to verify the advantage of employing DA in the strategy (see Figure S5 in the Supporting Information).

Reproducibility, Stability, and Selectivity of the miRNA Biosensor. The reproducibility of the ECL biosensor was detected by the variation coefficients (ECL signal) for three duplicate measurements of intra-assays and interassays. The relative standard deviation (RSD) of the detection were <5%. Therefore, the reproducibility and accuracy of the miRNA biosensor were available.

The stability of the ECL biosensor was evaluated under consecutive cyclic potential scans for 21 cycles at 0.5 pM of miRNA-155. The result is presented in Figure 5A; there was no significant change of the ECL response. The satisfying stability was ascribed to the probably reasons: First of all, the Au nanoparticles provided a high loading of DNA chains, which could increase the available chances of the chains and offer an efficient electron transfer tunnel. Simultaneously, the modified DNA chains kept its good bioactivity and superior conductivity.

From the practical point, selectivity was another essential element for miRNA exploration. As depicted in Figure 5B, the presence of 500 pM of thrombin aptamer (TBA), 500 pM of PDGF binding aptamer (PBA), 500 pM of miRNA-101, and 500 pM of miRNA-21 causes minimal ECL signal, which were similar to the blank test. When we tested miRNA-155 at a lower concentration (50 pM), there was a significant enhancement in the ECL intensity. Besides, the mixture in the presence of miRNA-155 (50 pM) with interference substances showed no significant changes, compared to the miRNA-155 solution. The above consequence illustrated the excellent specificity of the designed biosensor.

Application. To further investigate the feasibility and capability of our method, we further examined the expression of miRNA-155 in cell lysates, including a cervical cancer cell line (HeLa), human renal cubularepithelial cell line (HK-2), normal hepatocyte cell line (L02), and human umbilical vein endothelial cell line (HUVEC). We established the calibration curve for miRNA-155 detection (see Figures S2A and S2B in the Supporting Information). The expression level of miRNA-155 in different cell lysates then were analyzed based on the miRNAs extracted from the above cell lysates (see Figures S2C and S2D in the Supporting Information). The consequence illustrated that miRNA-155 had different expressions with different cells. The cell lysate from Hela had a higher expression level of miRNA-155. For comparison, the same samples were also measured by a commercial kit based on qRT-PCR (see Figure S3 in the Supporting Information). As can be seen in Figure 6, the results received using the ECL-based method were

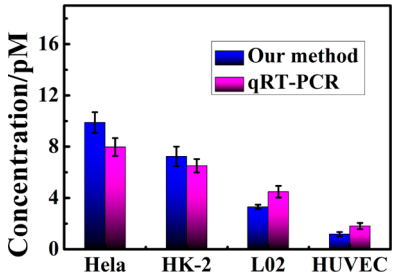

Figure 6. Real sample analysis of miR-155 detection in different cell lysates (Hela, HK-2, L02, and HUVEC). The error bars indicate the standard deviation of four analyses.

in good agreement with the qRT-PCR method. These results demonstrate that the proposed method provided an efficient and promising alternative tool for determining miRNA-155 in clinic analysis.

CONCLUSIONS

In summary, we demonstrated a highly sensitive off−on switching of ECL sensing platform for miRNA detection by coupling the DNAzyme-assisted target recycling and RCA, and our work has brought forth the following two novel ideas in our approach. First, our study provided an off−on switching sensor, which could be easily performed by strand displacement reactions, and first utilized DA to quench the luminol ECL signal, leading to the good stability and excellent specificity of the sensing system. Second, we designed a dual amplified strategy that has an ultrahigh sensitivity. The first amplification was initiated by Pb^{2+}-induced target recycling; thus, numerous cleaved DNA fragments were generated that acted as RCA primers to trigger the next amplification. With rational design, the RCA process generated several repeated sequences to bind with hemin, which remarkably enhanced the ECL signal of the luminol/H_2O_2 system. With outstanding reproducibility, stability, and selectivity, the proposed biosensor could be expanded on other interesting tumor markers.

ASSOCIATED CONTENT

Supporting Information

Additional electronic information as described in the essay. 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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