Supporting Information

Reusable electrochemiluminescence biosensor based on tetrahedral DNA signal amplification for ultrasensitive detection of microRNAs

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1. Reagents

Tris(2,2'-bipyridine)ruthenium dichloride ($Ru(bpy)_3^{2+}$) and hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4 \cdot 3H_2O$) were obtained from Shanghai Macklin Biochemical Co. Ltd. (Shanghai, China). Sodium borohydride ($NaBH_4$) and 6-mercapto-1-hexanol (MCH) were obtained from Sigma-Aldrich Chemicals Co., Ltd (Shanghai, China). 6X ficoll gel loading buffer and acrylamide were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). EDTA-2Na, potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate(II) ($K_4[Fe(CN)_6]$), potassium chloride (KCl), sodium dihydrogen phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4) were obtained from Nanjing Reagent Co. Ltd (Nanjing, China). $MgCl_2 \cdot 6H_2O$, Tris-magnesium acetate (TM) buffer, ammonium peroxodisulfate (APS) and orthoboric acid were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). N,N,N',N'-tetramethylethylenediamine (TEMED) and tripropylamine (TPA) were obtained from Shanghai Yien Chemical Technology Co., Ltd (Shanghai, China). The 0.1 M phosphate-buffered saline (PBS; pH 7.4) composed of K_2HPO_4, NaH_2PO_4, NaCl, and KCl were used as the supporting electrolytes in the ECL measurement process. Millipore water obtained from a Millipore water purification system (resistivity = 18.2 MΩ·cm) was used for all experiments.

2. Apparatus

Scanning electron microscopy (SEM) images were recorded on a Hitachi S-4800 scanning electron microscope (Hitachi Co., Japan), HRTEM images were taken by Tecnai G2 F20S-TWIN transmission electron microscope (Field Electron and Ion Co., United States), AFM images were recorded on Microscope MultiMode VIII (Bruker Co., USA), PAGE images were recorded on G:BOX F3 Gel Imager System (Synoptics Ltd., UK), ECL measurements were performed in a three-electrode system using an MPI-A ECL analyzer (Ruimai Co., China). The photomultiplier (PMT) voltage was -800 V. Cyclic voltammetry (CV) was carried out with a CHI600e instrument (CH Instrument Co., USA). Electrochemical impedance spectroscopy (EIS) was conducted

on a PARSTAT 2273 potentiostat/galvanostat (Advanced Measurement Technology Inc., USA) by applying an AC voltage amplitude of 5 mV in the frequency range from 0.1 Hz to 100 kHz in 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl.

3. Oligonucleotide Sequences

Table S1. Sequence information for the nucleic acids used in this study

Name	Sequences (5' - 3')	Modification
S1	CCACTGTGAATTCTCAACTGCCTGGT GATACGAGGATGGGCATGCTCTTCC CGACGGTATTGGACCCTCGCATG	N/A
S2	CGATTACAGCTTGCTACACGATTCA GACTTAGGAATGTTCGACATGCGAG GGTCCAATACCG	5'HS-SH C6
S3	CTACTATGGCGGGTGATAAAACGTG TAGCAAGCTGTAATCGACGGGAAGA GCATGCCCATCC	5'HS-SH C6
S4	TTTATCACCCGCCATAGTAGACGTAT CACCAGGCAGTTGAGACGAACATTC CTAAGTCTGAA	5'HS-SH C6
hairpin	CACTAAGACAGCGGAACTTAG	5'SH C6
R1	CAGCATCTAAGTTCCGCTGTCTTAG	N/A
R2	CTAAGACAGCGGAACTTAGATGCTG	N/A
miRNA-27a	UUCACAGUGGCUAAGUUCCGC	N/A
mRNA1	UUCACAGUGGCUAAGUUCCAC	N/A
mRNA2	UACACAGUGGAUAAGUUCCGC	N/A
mRNA3	UACACAGUGGCUAAGUUCCAC	N/A
mRNA4	GCTACACAGAGGCCATTGAAC	N/A

4. Preparation of TDN

The four ssDNA solutions were diluted to equal molar concentrations (2 μL, 50 μM) with TM buffer (20 mM Tris-HCl, 50 mM $MgCl_2$, and 100 mM KCl, pH 8.0) and mixed uniformly. The mixture was then placed in a polymerase chain reaction (PCR) thermocycle instrument, denatured at 95 °C for 5 min, and rapidly cooled to 4 °C within 30 s. After self-assembly, the prepared TDNs were stored at 4 °C until further use. The TDNs were characterized using 10 % polyacrylamide gel electrophoresis (PAGE) analysis.

5. Preparation of TDN-Ru(bpy)$_3^{2+}$ and miRNA-TDN-Ru(bpy)$_3^{2+}$

First, 10 μL TDN (1 μM) and 10 μL Ru(bpy)$_3^{2+}$ (10 mM) were mixed into 380 μL TM buffer and stored at 4°C overnight to allow Ru(bpy)$_3^{2+}$ to be attached to TDN as ECL signal label by electrostatic adsorption. The solution was then purified using an Amicon Ultra 3K unit and centrifuged to filter the uncombined Ru(bpy)$_3^{2+}$. Finally, TDN-Ru(bpy)$_3^{2+}$ (TDN-Ru) was stored at 4 °C until further use. MiRNA-TDN- Ru(bpy)$_3^{2+}$ (miR-TDN-Ru) were obtained by mixing equal volumes (50 μL) TDN-Ru (50 nM) with miRNA-27a (1 nM) and incubating at 4 °C for more than 3 h.

6. Preparation of NiFe-LDHs and NiFe-LDH@AuNPs

NiFe-LDHs were synthesized according to the previous literature.[1] First, $Ni(NO_3)_2 \cdot 6H_2O$ (2 mM) and $Fe(NO_3)_3 \cdot 9H_2O$ (2 mM) were dissolved in 60 mL H_2O to form a homogeneous solution. Subsequently, 4 mM ammonium fluoride and 10 mM urea were added to above solution to form a uniform mixture. Then, the mixture was reacted at 120 °C for 6 h. Finally, NiFe-LDHs were obtained after centrifugation, cleaning and drying. The synthesis of NiFe-LDH@AuNPs mainly involved the following steps: first, 100 μL NiFe-LDH (2 mg/mL) was dispersed in 2 mL H_2O. Subsequently, 50 μL $HAuCl_4$ (1%) and 40 μL freshly prepared $NaBH_4$ (3.8 mg/mL) were successively added during the stirring process. The resulting purple red solution

was centrifuged at 13,000 rpm. Finally, the prepared NiFe-LDH@AuNPs were washed and dispersed in 1 mL H_2O for subsequent use.

7. ECL Biosensor Fabrication

First, bare GCE was polished and cleaned to a mirror surface. Subsequently, 10μL NiFe-LDH@AuNPs were added to the electrode and then the hairpin DNA was bonded to NiFe-LDH@AuNPs modified electrode via the Au-S bond. After 1 h, the electrode was softly washed with PBS. To block the non-specific binding sites, 10 μL of MCH was covered onto the modified electrode. After 30 min, the electrode was rinsed with PBS. Next, 5 μL of miR-TDN-Ru was dropped onto the modified electrode surface for 2 h at 37 °C, and the biosensor was obtained by carefully rinsing with PBS buffer thrice. In order to achieve electrode regeneration, 10 μL R1 was introduced to the electrode surface, incubated for 1 h at 37 °C, and gently washed with PBS. Finally, 10 μL R2 was added, incubated for 2 h at 37 °C, and gently rinsed with PBS to make the electrode surface return to the initial state with hairpin DNA modification, realizing the reuse of the same electrode.

8. ECL Measurement

Before measurement, TDN-Ru was incubated with different concentrations of miRNA-27a standard solution, and then assembled with modified electrode to form a trinity structure ECL biosensor. After washing, the resultant biosensors were performed in 0.1 M PBS containing 0.1 M TPrA (pH 7.4) to investigate the ECL signal. The scanning potential was 0-1.3 V with a scanning rate of 0.1 V/s, and a voltage of -800 V for the photomultiplier.

9. CV curves of NiFe-LDH and NiFe-LDH@AuNPs
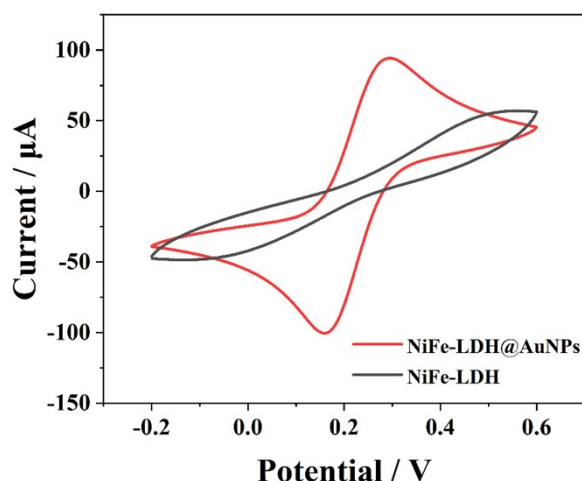
Fig. S1. CV curves of NiFe-LDH and NiFe-LDH@AuNPs in 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture containing 0.1 M KCl. The electrochemical performance of NiFe-LDH and NiFe-LDH@AuNPs modified electrodes were explored to verify the effect of AuNPs loading on NiFe-LDH. Compared with the NiFe-LDH modified electrode, the current of NiFe-LDH@AuNPs modified electrode increased remarkably, indicating that the introduction of AuNPs can significantly accelerate the electron transfer.

10. ECL intensity of NiFe-LDH and NiFe-LDH@AuNPs modified electrode
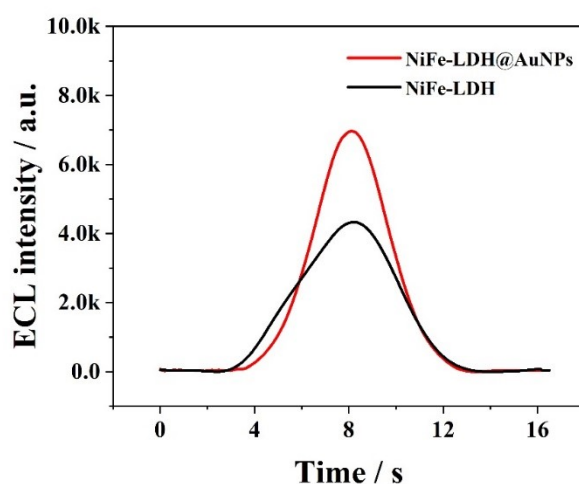
Fig. S2. ECL intensity of NiFe-LDH and NiFe-LDH@AuNPs in 0.1 M pH 7.4 PBS containing and 5 nM $Ru(bpy)_3^{2+}$ and 0.1 M TPrA. Scan range: 0.2-1.3 V, scan rate: 0.1 V/s. PMT voltage: -800 V. The results of ECL test of NiFe-LDH and NiFe-LDH@AuNPs modified electrode also proved that the NiFe-LDH composite loaded with AuNPs is more helpful to improve the ECL performance.
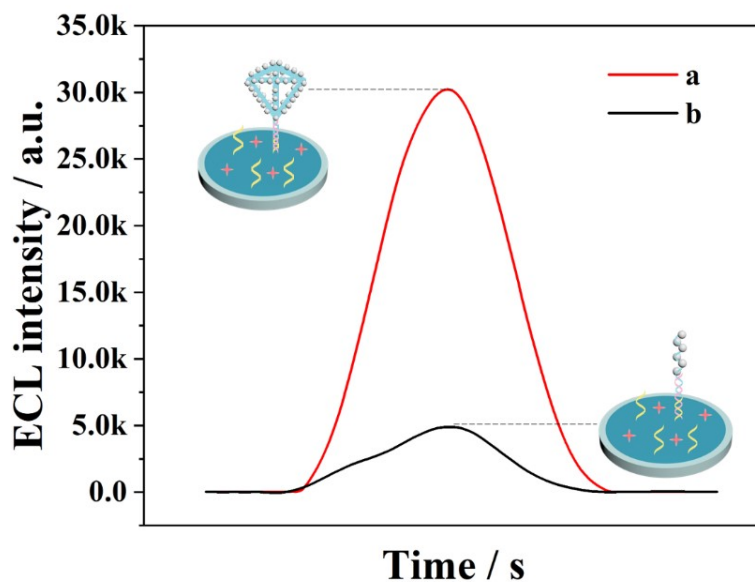
11. ECL Stability of TDN-Ru
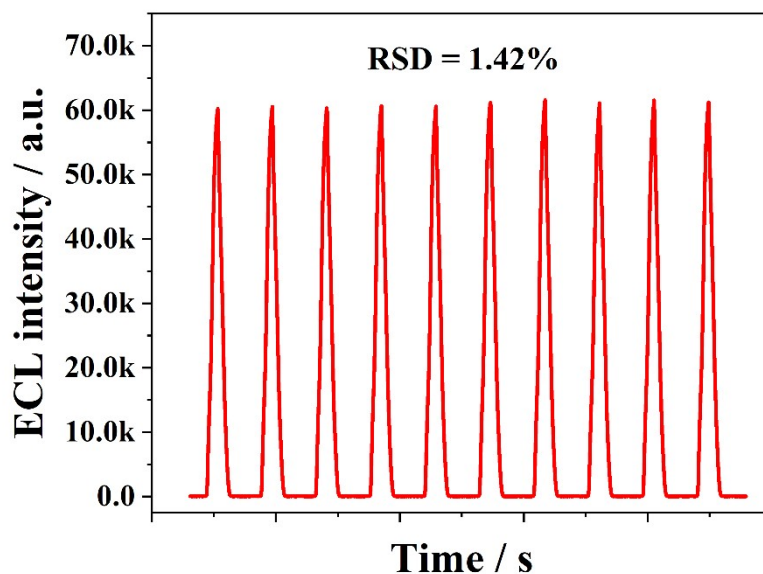
Fig. S3. Stability of the GCE modified with 50 nM TDN-Ru under 10 repetitive cyclic potential scans. Scan range: 0.2-1.3 V, scan rate: 0.1 V/s. PMT voltage: -800 V.

## 12. ECL response of the biosensor with different signal probe

Fig. S4. ECL response of the biosensor with different signal probe: (a) TDN as signal probe, (b) ssDNA as signal probe. Scan range: 0.2-1.3 V, scan rate: 0.1 V/s. PMT voltage: -800 V. The effect of different response probes on ECL for the biosensors was examined to confirm the advantage of

TDN probe. Compared to that of the single DNA signal probe, the ECL intensity of the biosensor constructed with the TDN signal probe is approximately 6 times stronger than that of the single DNA probe, indicating that the TDN can confer superior ECL performance for the biosensor. This phenomenon could attribute to the fact that TDN not only load abundant $Ru(bpy)_3^{2+}$ emitter, but also has stable spatial structure than ssDNA, reducing strand entanglement on the electrode surface and thus improve the hybridization efficient.
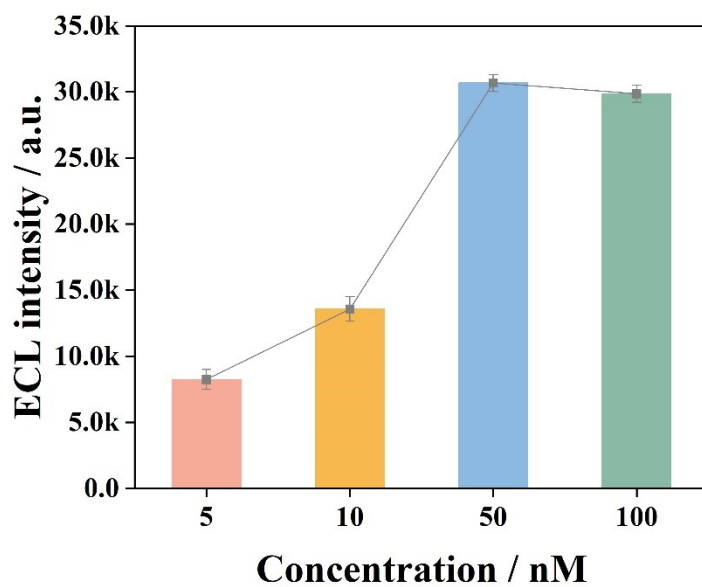
13. Condition optimization of ECL experiment
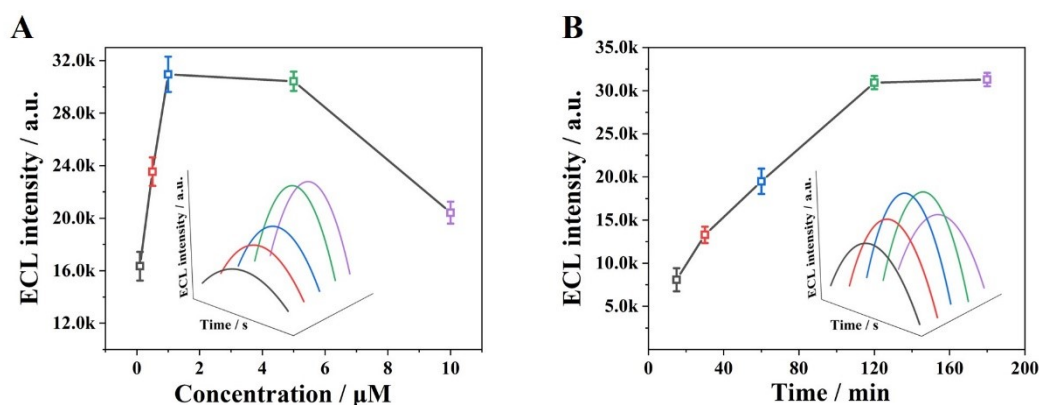
Fig. S5. (A) Effect of hairpin DNA concentration (0.1 μM, 0.5μM, 1 μM, 5 μM, and 10 μM) on ECL intensity, (B) Effect of incubation time (15, 30, 60, 120, and 180 min) of miR-TDN-Ru on ECL intensity. The performance of the ECL biosensor was considerably affected by the concentration of hairpin DNA immobilized on the electrode surface. To determine the optimal experimental performance, the hairpin DNA concentration was investigated. With an increase in hairpin DNA concentration, the ECL intensity of the biosensor gradually increased, after the concentration was exceeded 1 μM, the ECL signal decreased sharply. This phenomenon could be attributed to the "steric hindrance effect" caused by excess hairpin DNA jammed and entangled on the electrode surface. Therefore, 1 μM was used as the optimal concentration of hairpin DNA in this work. In addition, the incubation time of miR-TDN-Ru on the proposed electrode was also discussed. As displayed in Fig. 4B, the ECL intensity gradually increased with the increasing incubation time, after, it remained constant at 120 min, indicating that the reaction has been completed at the optimum incubation time of 120 min.
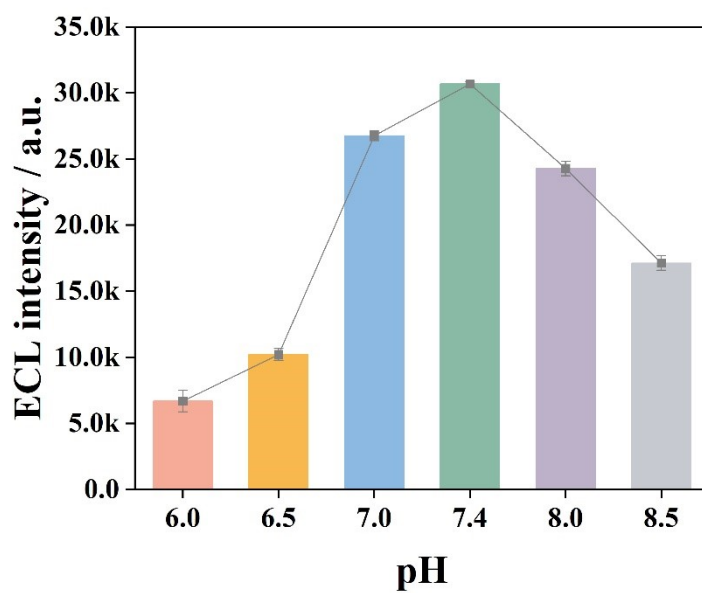
Fig. S6. Effect of different pH values of PBS (6.0, 6.5, 7.0, 7.4, 8.0 and 8.5) on ECL intensity. pH 7.4 of PBS was selected as the optimal experimental condition.


Fig. S7. Effect of TDN-Ru probe concentration (5 nM, 10 nM, 50 nM, 100 nM) on ECL intensity. 50 μM TDN-Ru signal probe was selected as the optimal experimental condition.

14. Comparison with other reported methods for detection of miRNA

Table S1. Comparison of this work with other reported methods for detection of miRNA

Detection Methods	Linear range	LOD	References
fluorescence	20 pM to 10 nM	8 pM	2
electrochemical	1 fM to 10 nM	0.31 fM	3
surface enhanced raman scattering	12 fM to 18 pM	5 fM	4
ECL	1 fM to 1 nM	0.13 fM	5
ECL	1 fM to 10 nM	0.33 fM	6
ECL	100 aM to 1 nM	11.7 aM	this work

## 15. Effect of concentrations of DNA R1 and R2 on regeneration experiment
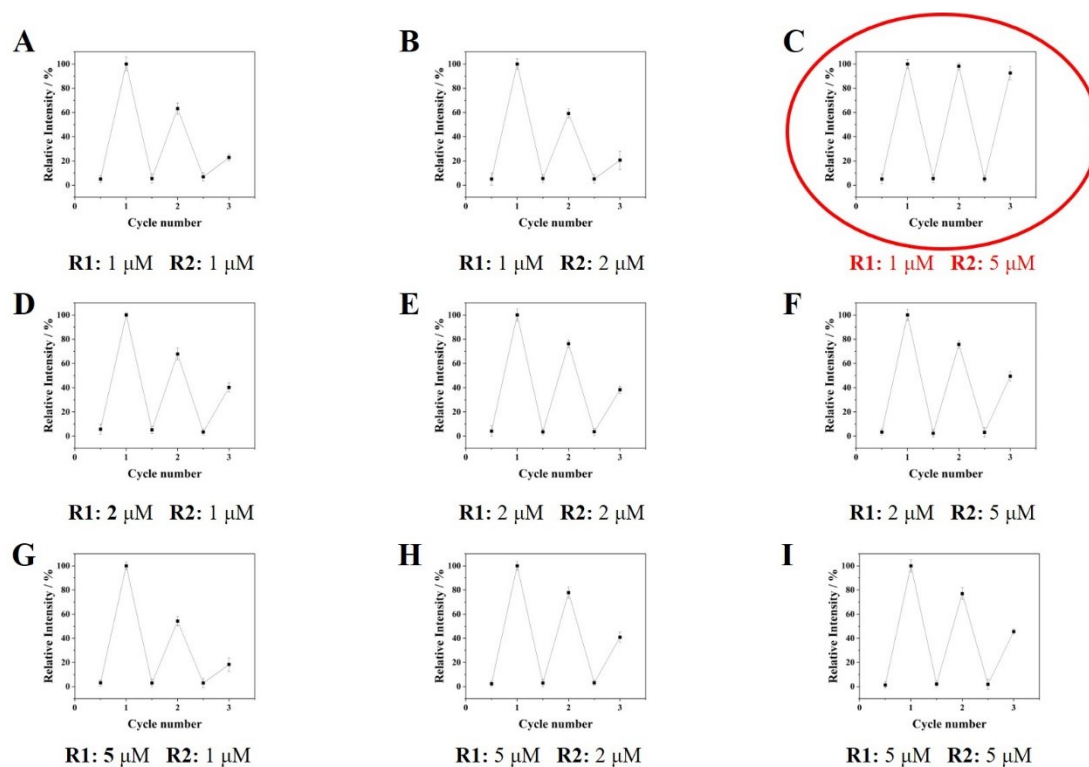
Fig. S8. Effect of different concentrations of DNA R1 and R2 on electrode regeneration experiment. (A) R1: 1 μM, R2: 1 μM, (B) R1: 1 μM, R2: 2 μM, (C) R1: 1 μM, R2: 5 μM, (D) R1: 2 μM, R2: 1 μM, (E) R1: 2 μM, R2: 2 μM, (F) R1: 2 μM, R2: 5 μM, (G) R1: 5 μM, R2: 1 μM, (H) R1: 5 μM, R2: 2 μM, (I) R1: 5 μM, R2: 5 μM. Scan range: 0.2-1.3 V, scan rate: 0.1 V/s. PMT voltage: -800 V. The results showed that with the increase of the ratio of R2 to R1 chain concentration, the signal level recovered within 3 regenerations increased accordingly. This may be due to the fact that when

the replacement chain concentration is higher, there will be more reaction opportunities and higher probability of completing the reaction in the reaction system, and the signal recovery after regeneration is better. Therefore, 1 μM R1 and 5 μM R2 were selected as the optimal experimental conditions.

16. ECL intensity of the regenerated biosensor
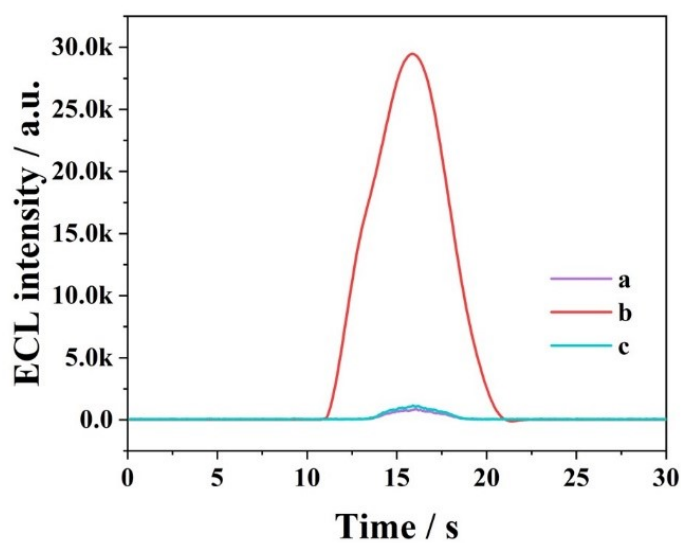
Fig. S9. ECL intensity of the regenerated biosensor: the "off" state of the biosensor (curve a), "on" state of the biosensor (curve b), and regeneration of the biosensor with the representation of "off" state (curve c).

17. Stability of the ECL biosensor.
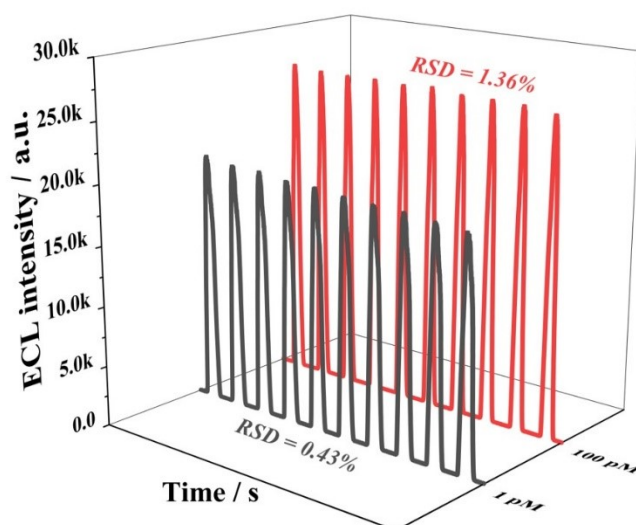
Fig. S10. Stability of the proposed biosensor with 1 and 100 pM miRNA-27a. Working solution: 0.1 M pH 7.4 PBS containing 0.1 M TPrA. Scan range: 0.2-1.3 V, scan rate: 0.1 V/s, PMT voltage: -800V.

18. Recovery experiments for miRNA-27a in real human serum

Table S2. Recovery experiments for miRNA-27a in real human serum

Sample	Added (fM)	Found (fM)	Recovery (%)	RSD (n = 3, %)
1	10	9.7	97.1	4.3
2	100	95.4	95.4	1.6
3	1000	966.7	96.7	2.6

19. References

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