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Sensitive electrochemiluminescence analysis of lung cancer marker miRNA-21 based on RAFT signal amplification

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1 Chemicals

N-acryloxysuccinimide (NAS), luminol and 6-mercapto-1-hexanol (MCH) were purchased from Sigma-Aldrich (St. Louis, USA). 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPAD) were purchased from Aladdin Bio-Chem Technology Company (Shanghai, China). Zirconium dichloride oxide octahydrate ($\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$) and azobisisobutyronitrile (AIBN) were made in J&K Scientific Co. Ltd. (Shanghai, China). N,N-Dimethylformamide (DMF), absolute ethanol ($\text{C}_2\text{H}_5\text{OH}$). Normal human serum purchased from Beijing Solarbio Science & Technology Co., Ltd. Thiolated PNA (**3'-SH-ATC GAA TAG TCT GAC TAC AAC T-5'**) was Thiolated peptide nucleic acid (PNA) was custom-made by Panagene Inc. (South Korea). Target miRNA-21 (**UAG CUU AUC AGA CUG AUG UUG A**), single-base-mismatched (1MT) (**UAG CUU AUC GGA CUG AUG UUG A**), three-base-mismatched (3MT) (**UUG CUU AUC GGA CUG AUC UUG A**), miRNA-141(**UAA CAC UGU CUG GUA AAG AUG G**), miRNA-155 (**UUA AUG CUA AUC GUG AUA GGG GU**) were supplied by Sangon Biotech Company, Ltd. (Shanghai, China).

2 Apparatus

The electrochemical and ECL measurements were conducted on an MPI-E multifunctional electrochemical and ECL analytical system (Xi'an Remex Analytical Instrument Ltd. Co., China). All ECL measurements were conducted in a 10 mL glass cell. Gold electrode ($\phi = 2.0$ mm) was used as working electrode, an Ag/AgCl (saturated KCl solution) was used as reference electrode and platinum wire was used as counter electrode (Chenghua, Shanghai, China). The voltage of the photomultiplier tube (PMT) during detection was set at 900 V. The cyclic voltammogram (CV) and the electrochemical impedance spectroscopy (EIS) were analyzed using a CHI-660E voltammetric analyzer (Shanghai Chenhua Apparatus Inc, China) containing 0.5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$. Atomic force microscopy (AFM) was purchased from Seiko Instruments Inc-SII (instrument model: SPA-300HV90, Japan), Transmission electron microscopy (TEM) measurements were carried out using JEM-2100 high resolution-TEM (JEOL, Japan).

3 Experimental section

3.1 The pretreatment of Au electrode. The surface of the gold electrode need a pretreatment before being modified. First, electrode surface was polished to be specular with 0.05 μm alumina slurries. Then, it was cleaned ultrasonically in ultrapure water and absolute ethanol. After this, it was

immersed in piranha solution (V (98 % H_2SO_4): V (30 % H_2O_2) = 3:1) for 30min to thoroughly remove impurities, and then rinsed with anhydrous ethanol and ultrapure water in sequence to clear the residual piranha solution. Afterwards, cyclic voltammetry (CV) was used to electrochemically pretreat the electrode surface in electrolyte solution (0.5 M H_2SO_4) in the potential range of -0.2 V to 1.5 V at scan rate is 0.05V/s, and this operation continued until cyclic voltammogram was steady. Ultimately, the electrode surface was purged by sonication in anhydrous ethanol and ultrapure water in succession and was dried with N_2 .

3.2 Fabrication of the ECL sensor. Firstly, the gold electrode need to be polished and cleaned before use, the pretreatment process was recorded in the supporting information. Subsequently, 10 μ L peptide nucleic acid (PNA) solution (0.5 μ M) was dropped onto the clean electrode surface at 37 °C. After two hours' incubation, ultrapure water was used to rinse the nonspecific adsorbed PNA. Then MCH (prepared by 40% C_2H_5OH) was used to block the remaining active sites for 0.5 h. Next, 10 μ L miRNA-21 (pH 8.0, PBS) was dropped onto the electrode for specific recognition with PNA. Two hours later, the non-specific adsorbed miRNA-21 was rinsed with PBS. After that, the electrodes were immersed in 5.0 mM $ZrOCl_2$ solution (40% C_2H_5OH) for 40 min, the Zr(IV) ions were introduced on the electrode surface by the conjugation of phosphate with Zr(IV) ions. Later, the electrodes were incubated in 5.0 mM CPAD solution, the chain transfer agent of RAFT, to form phosphate-Zr(IV)-carboxylate structure for 40 min at 37 °C. Afterwards, the electrode was incubated in SI-RAFT solution (5% DMF, 0.5 mM NAS, 75 μ M AIBN) at 55 °C for polymerization reaction. Two hours later, the non-specific adsorbed materials on the electrode surface were washed away by DMF and ultrapure water. Finally, the electrode was placed in luminol solution to react fully with the functional groups of monomer NAS on the polymer chain for 4 hours. After washing with DMF and ultrapure water, the proposed biosensor was constructed.

3.3 ECL detection of miRNA-21. The as-prepared sensors were soaked into 10 mL PBS buffer (0.2 M, pH 7.5, containing 20 μ L H_2O_2). The voltage of the photomultiplier tube was set at 900 V. (Cyclic voltammetry parameter setting: scan rate 100mV/s, quiet time: 5 s, the potential ranging from -0.2 to 0.6 V.)

3.4 Cyclic voltammetry characterization

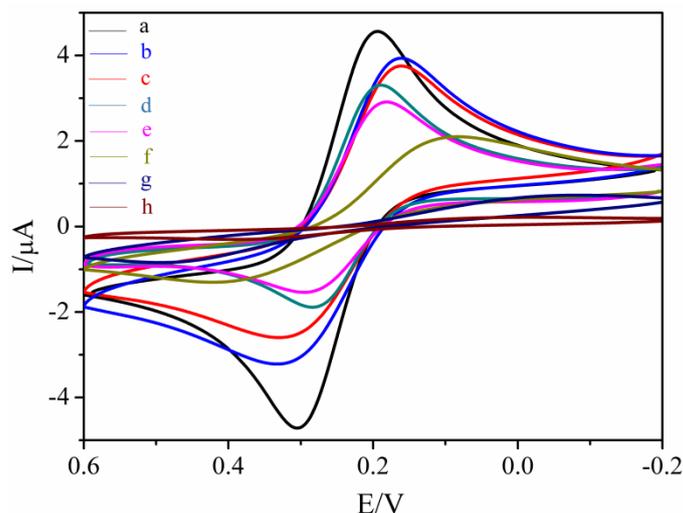


Fig. S1 Cyclic voltammeters of the bare Au electrode (a), PNA (b), PNA/MCH (c), PNA/MCH/miRNA-21 (d), PNA/MCH/miRNA-21/Zr⁴⁺ (e), PNA/MCH/ miRNA-21/Zr⁴⁺/CPAD (f), PNA/MCH/miRNA-21/Zr⁴⁺/CPAD/ SI-RAFT polymer chain (g), PNA/MCH/miRNA-21/Zr⁴⁺/ CPAD/SI-RAFT polymer chain/luminol (h) modified with Au electrode.

3.5 TEM characterization. The sensors prepared in accordance with section 3.2 was immersed in 0.5 mL ethanol and water with 1:1 solution for 3 min with ultrasonic. After that, the solution was immediately dropped on a copper net and TEM was performed after drying. The accelerating voltage was 200kV.

3.6 AFM characterization

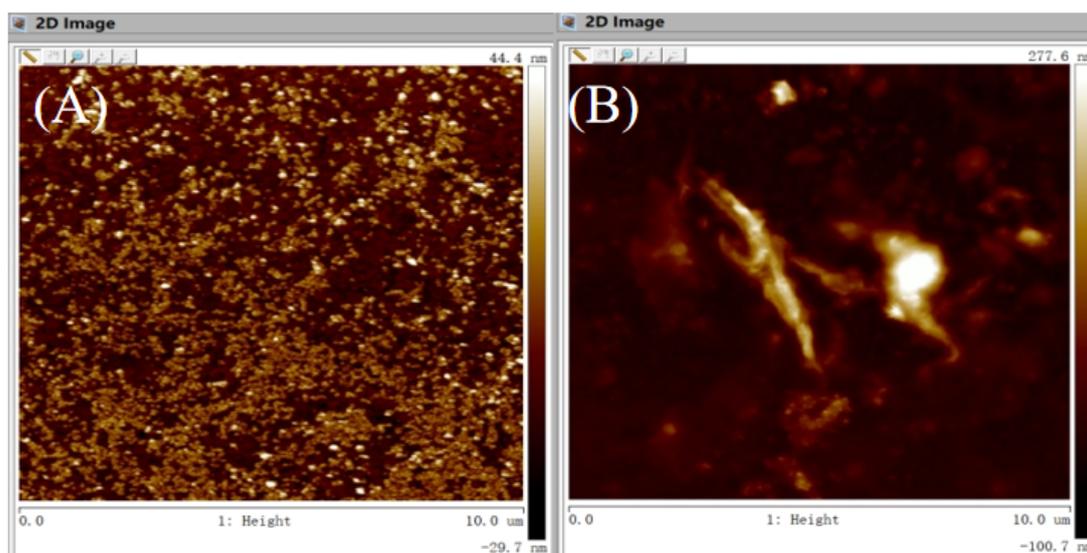
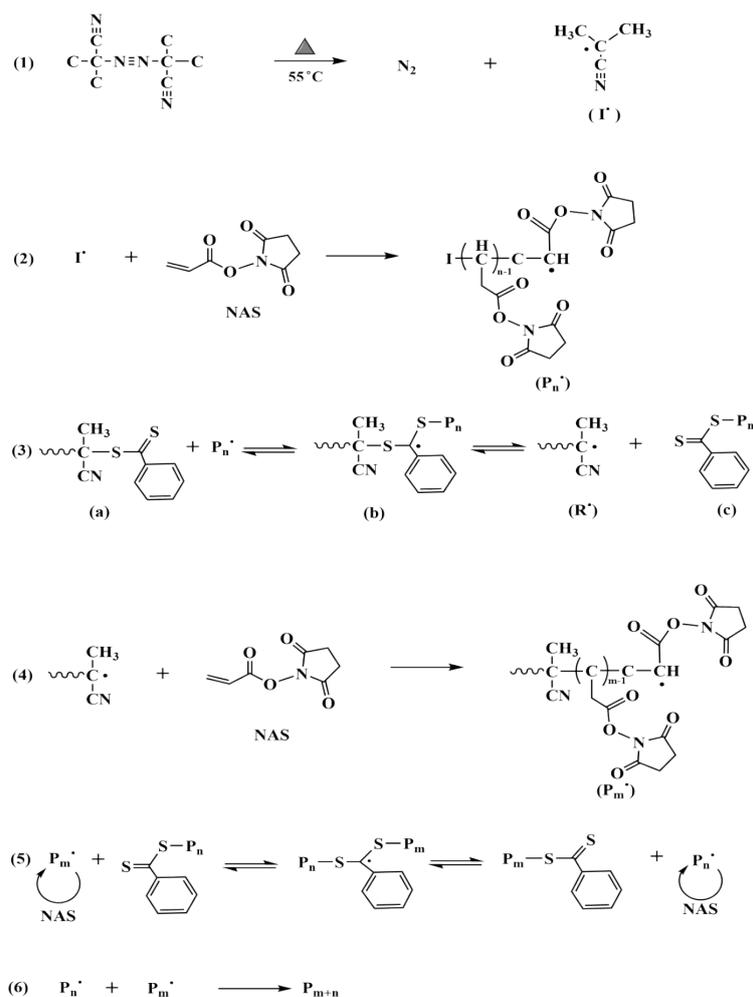


Fig. S2 The AFM images (2D) of the electrode before (A) and after (B) SI-RAFT reaction.

4 The principle of NAS SI-RAFT reaction. As shown in Scheme S1, the thermally initiated SI-RAFT polymerization was divided into the following steps (1) When the temperature was raised to 55°C, the C-N bond of initiator AIBN is broken to produce alkyl radicals I· and nitrogen. (2) Alkyl radicals attack C=C bond of monomer (NAS) to produce a new chain free radical P_n·. (3) The chain transfer agent (CPAD, a) can quickly capture free radicals in the polymerization system to form stable free radicals (b). This free radical does not initiate polymerization of the monomer, but rapidly split to form a new surface-tethered radical R· and the dormant chains (c). (4) R· can initiate polymerization of the NAS monomer to form a chain radical P_m·. (5) P_m· can be quickly captured by the thiocarbonylthio-group-capped dormant chains. In this way, every site of chain transfer agent is tethered to a long polymer chain. (6) The polymerization is terminated by the combination of the chain radical P_m· and P_n·.



Scheme S1. The mechanism of thermally initiated SI-RAFT reaction

5. Optimization of experimental conditions. In order to achieve the best analytical performances, the experimental conditions which may affect the analytical performance of sensor were optimized. The thermal decomposition of initiator AIBN is critical to SI-RAFT polymerization. The low concentration of AIBN makes polymerization rate slow. On the contrary, if the concentration of AIBN is too high, excess alkyl radicals will be generated, which occupy the active site on the electrode surface and hinder the formation of polymer chain. Fig. S1 (A) shows the ECL intensity reached a peak value when the concentration of AIBN was 75 μM . So 75 μM AIBN was confirmed as the optimal concentration.

According to the principle of polymerization, the aggregation chain will grow over time until an equilibrium is reached. In this case, it is indispensable to investigate the optimal polymerization time. It can be observed that the ECL signal grows slowly and even no longer grows over time after 2h from Fig. S1 (B). Thus, 2h was selected as the optimal SI-RAFT polymerization time.

Because the electrochemical signal comes from luminol molecules that react with NAS polymers, the incubation time in the luminol solution is a critical factor which may affect the ECL intensity. It can be observed from Fig. S1 (C) that the ECL intensity increased with time in the first 4 hours, and further extension of incubation time did not result in a significant increase in signal, so we used 4 hours as the incubation time for luminol in subsequent studies.

The influence of pH value of PBS and H_2O_2 content on the experiment was also evaluated. The results were shown in Fig. S1 (D) and Fig. S1 (E). We selected the pH of 0.2 M PBS buffer as 7.5. The ECL intensity rapidly increased with the H_2O_2 content, yielding a peak value at 20 μL . While excessive H_2O_2 will lead to an increase in the baseline of ECL signal, and the ECL intensity exceeds the instrumental range. Consequently, we set the H_2O_2 addition amount as 20 μL in the following experiments.

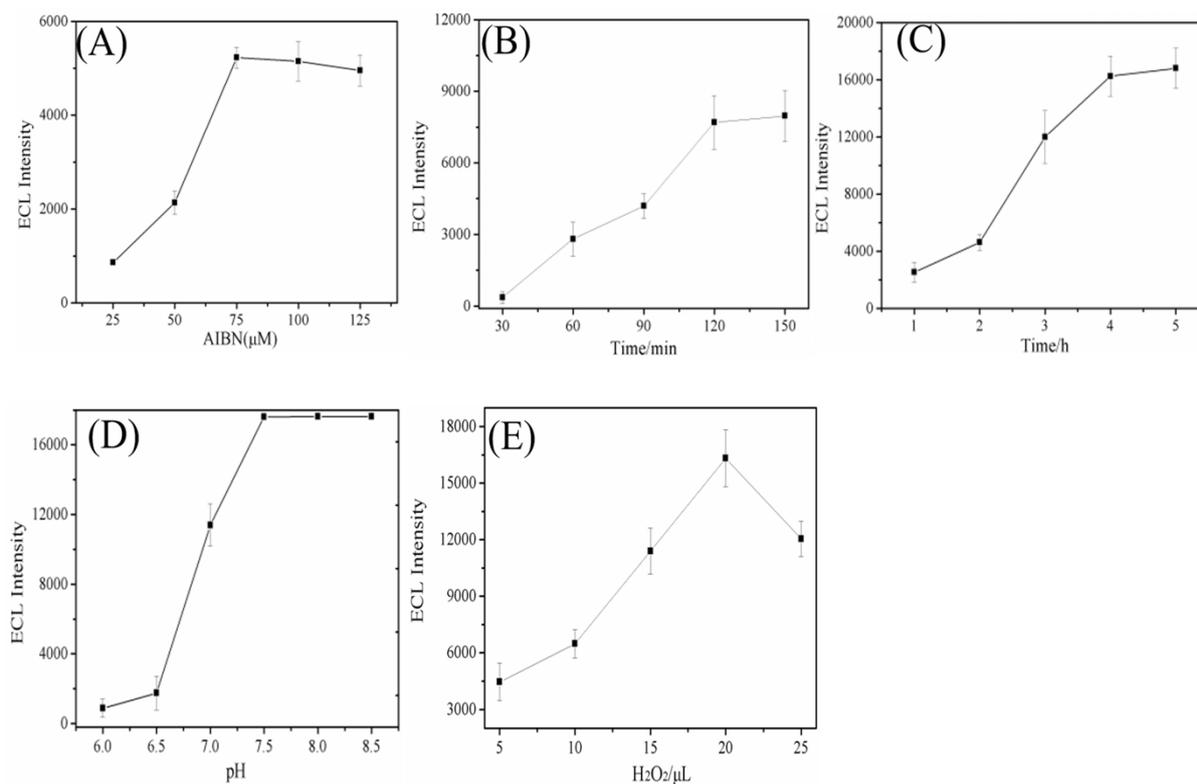


Fig. S4. Optimization of the experimental conditions. (A) Effect of the concentration of AIBN. (B) The effect of SI-RAFT polymerization reaction time. (C) The effect of the incubation time of luminol (D) The effect of the pH of PBS buffer (E) The effect of the content of H_2O_2 .

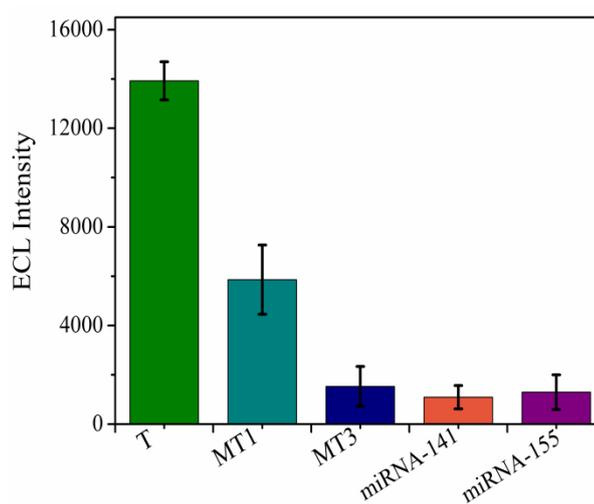


Fig. S5. The specificity of the ECL sensor for microRNA-21(1 pM) and interferent miRNAs (MT1, MT3, miRNA-141 and miRNA-155 were 1nM).

Table S1. Comparison of the analytical performance of our method with other methods.

method	linear range	LOD	ref.
Electrochemical	1 fM - 10 pM	1 fM	1
Surface-Enhanced Raman Scattering	1 fM - 100 pM	393 aM	2
Electrochemical	0.02 pM–10 pM	8.2 fM	3
Electrochemiluminescence	1.0 fM - 1.0 nM	0.5 fM	4
Electrochemical	1 aM - 1 nM	0.32 aM	5
Electrochemical	1.0 fM - 1.0 nM	0.34 fM	6
Electrochemiluminescence	10 aM - 1 pM	0.21 aM	This work

Table S2. Detection of miRNA-21 in human serum sample

Samples	Found	Added	Total Found	RSD % (n=3)	Recovery
1	11.3 aM	0.2 fM	0.21 fM	3.23	99.4%
2	10.9 aM	2 fM	2.25 fM	6.75	111.9%
3	11.7 aM	20 fM	18.46 fM	8.94	92.2%

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