Supporting Information

Ultrasensitive Electrochemiluminescence Biosensor for Nuclear Factor kappa B p50 based on Proximity Hybridization-induced Hybridization Chain Reaction

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1. Materials and Instruments

1.1. Materials and reagents

Dichlorotris (1,10-phenanthroline) ruthenium (II) hydrate ($Ru(phen)_3^{2+}$), 1,4-dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH) and Tripropylamine (TPA) were provided by Sigma-Aldrich (St. Louis, MO, USA). Lambda exonuclease (λ exo) and its reaction buffer were purchased from Thermo Fisher Scientific (Shanghai, China). The purified human recombinant NF-κB p50 was bought from Cayman Chemical (Ann Arbor, MI, USA). Human GP73 (GP73), alpha fetoprotein (AFP), carcino embryonic antigen (CEA) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Oridonin was purchased from Solarbio (Beijing, China). All oligonucleotides used in this work were provided by Sangon Inc (Shanghai, China) and their sequences are shown as follows:

5'P-DNA1: 5'-P-CACCTGGAAAGTCCCGATCCCACAC-CCTACT-3'

DNA2: 5'-GCCTTTACGCCAATATCGGGACTTTCCAG-GTG-3'

HP1: 5'-CGCCACCCACACCTGCTAGTAGTAGGGTGT-GGGTGGCGTAAAGGC-3'

HP2: 5'-ACTAGCAGGTGTGGGTGGCGGCCTTTACGC-CACCCACACC-3'

HP1-SH: 5'-CGCCACCCACACCTGCTAGTAGTAGGGTGTGGGTGGCGTAAAGGCCCCCCC-$(CH_2)_6$-SH -3'

Buffer used in this study:

Tris-HCl buffer: 10 mM Tris, 10 mM NaCl (pH 7.5).

Reaction buffer: 10 mM Tris, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM DTT (pH 7.5).

Binding buffer: 10 mM Tris, 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg/mL yeast tRNA, 0.25 mM DTT (pH 7.5).

1.2. Instruments

The electrochemical and ECL intensity were detected by an electrochemical workstation (CHI 660D, Chenhua Instruments, Shanghai, China) and a BPCL Ultra-Weak Luminescence Analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China), respectively. Gel electrophoresis was conducted using a DYY-8C electrophoretic apparatus (Beijing Liuyi Instrument Factory, Beijing, China) All ECL measurements were performed on a three-electrode system with Ag/AgCl (3.0 M KCl) as the reference electrode, platinum wire electrode as the counter electrode, and gold electrode (diameter = 3 mm) as the working electrode.

2. Preparation of HCR Amplification Solution

10 μM 5'P-DNA1 and 10 μM DNA2 were mixed in equal volumes, and the mixture was incubated at 90 ℃ for 10 min, and then slowly cooled to room temperature to obtain dsDNA. The above 5'P-dsDNA (5 μM, 4 μL) was then mixed with different concentrations of the target NF-κB p50 in protein binding buffer (10 μL) and incubated at 37 °C for 30 min. Subsequently, 20 U λ exo and its buffer were added for 30 min digestion at 37 °C, and the enzyme was inactivated at 75 °C for 20 min. Finally, hairpin HP1 (10 μM, 10 μL) , HP2 (10 μM, 12.5 μL), $Ru(phen)_3^{2+}$ (1 mM, 10 μL) and a certain amount of reaction buffer were added to make a final total volume of 60 μL, and incubated at 37 °C for 105 min for HCR amplification and to ensure that $Ru(phen)_3^{2+}$ was embedded in the dsDNA grooves. The amplification solution was reserved for

further use.

3. Modifications of Gold Electrodes and ECL Detection of NF-κB p50

Before modification, the gold electrodes (3 mm in diameter) were polished sequentially with 0.3 μm and 0.05 μm alumina powder and then sonicated in ethanol and ultrapure water, respectively. Next, the gold electrode was immersed in 0.5 M sulfuric acid (H_2SO_4), and the cyclic voltammetry curve was scanned in the potential range of 0 ~ 1 V until a stable cyclic voltammogram with three distinct oxidation peaks and one reduction peak position was obtained. The activated gold electrode was removed and rinsed with ultrapure water for further use. The hairpin probe HP1-SH1 (1 μM, 10 μL) was covalently bound to the gold electrode surface by the Au-S bond. To reduce non-specific adsorption, 1.0 mM MCH was added dropwise to the electrode surface and allowed to stand at 37 °C for 45 min to block any nonspecific binding sites. Finally, the modified gold electrode was immersed in the previously prepared HCR amplification solution, and incubated at 37 °C for 1 h. The gold electrode treated in each of the above steps was rinsed with ultrapure water to remove excess unreacted and non-specifically adsorbed material.

The ECL assay electrolyte was 2 mL phosphate buffer (6.7 mM, pH 7.4) containing 20 mM TPA. The photomultiplier tube was set to -800 V and ECL measurements were made with a potential range of 0.2 V to 1.6 V and a scan rate of 50 mV/s. All experiments were repeated three times. Average peak ECL intensity was reported for all experiments.

4. Gel Electrophoresis.

The sensor was characterized by 12% polyacrylamide gel electrophoresis. The running buffer was 0.5× TBE (pH 8.2), the applied voltage was 80 V, and electrophoresis was carried out for 120 min at room temperature.

A fluorescent dye was prepared by mixing Super Green (100×) with loading buffer at a volume ratio of 1:1. The DNA reaction product and the dye were mixed in a volume ratio of 2:1, and incubated at room temperature for 15 min before the mixture was applied.

5. Impedance Detection..

Impedance analysis was performed using a CHI660D electrochemical analyzer with an electrolyte of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ (containing 100 mM KCl), operating at a voltage of 0.258 V and an impedance frequency range of 10^{-1} ~ 10^4 Hz.

Figure S1

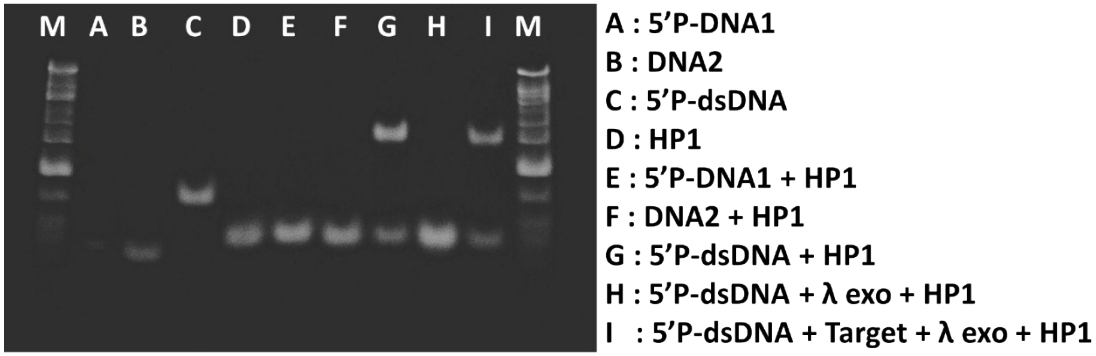
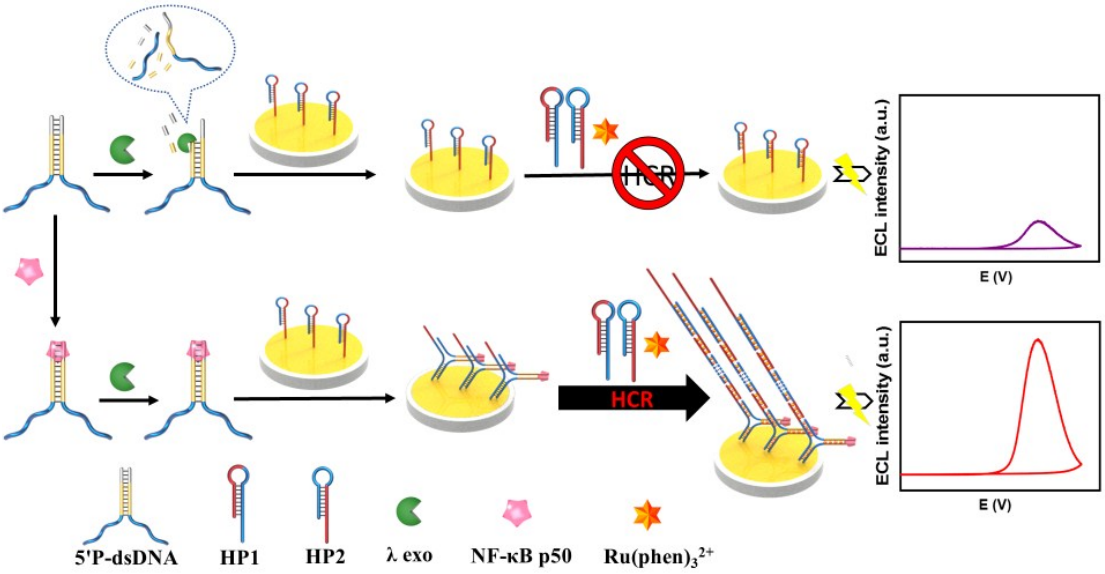


Fig. S1 Polyacrylamide gel electrophoresis analysis of the proximity hybridization (M: Marker).

Scheme. 2



Scheme 2 The principle of the on-surface-HCR-based ECL biosensor for NF-κB p50

Figure S2

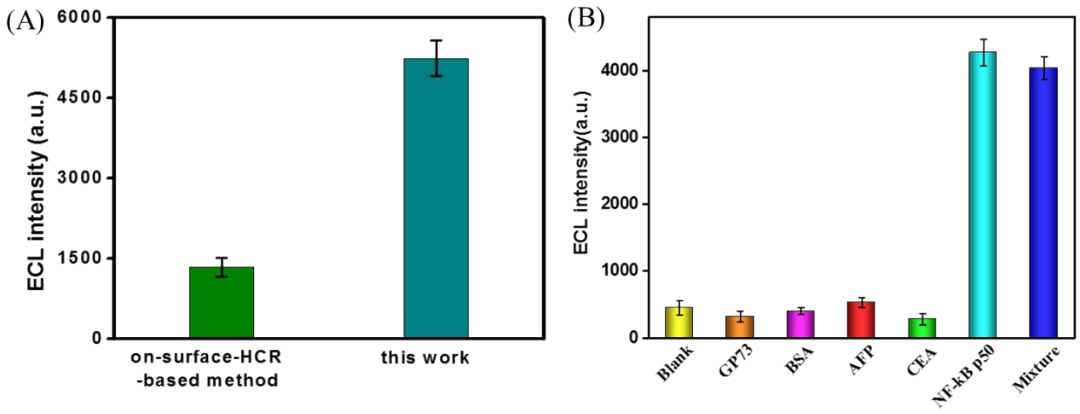


Fig.S2 (A) Comparison of ECL intensity between the on-surface-HCR-based method and the proposed method in the presence of 500 pM NF-κB p50. (B) ECL response in the presence of 100 pM NF-κB p50 or 10 nM of other analytes. All the detection was performed in 6.7 mM phosphate buffer (pH 7.4) containing 20 mM TPA. Scan range: 0.2 to 1.6 V. Scan rate: 50 mV/s.

Figure S3

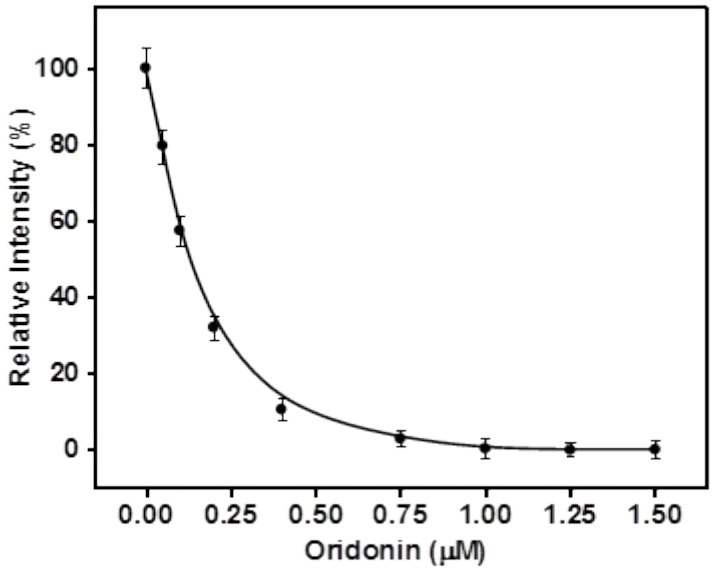


Fig.S3 The inhibition effect of oridonin on the ΔECL (different concentrations of oridonin were incubated with 500 pM of NF-κB p50). The detection was performed in 6.7 mM phosphate buffer (pH 7.4) containing 20 mM TPA. Scan range: 0.2 to 1.6 V. Scan rate: 50 mV/s.

Table S1 Comparison of the proposed method with the reported method for the detection of NF-κB p50

Method	Dynamic range	Detection limit
Fluorescence[1]	50 pM to 1 nM	45.6 pM
Square Wave Voltammetrys[2]	200 pM to 1.0 nM	40 pM
Chronocoulometry[3]	100 pM to 10 nM	80 pM
Electrochemical Impedance Spectroscopy[4]	10 pM to 20 nM	7 pM
The on-surface-HCR-based method	100 pM to 50 nM	30 pM
The proposed method	0. 5 pM to 300 nM	0.2 pM

Table S2 Results of the recovery test of NF-κB p50 in HeLa cell lysates (n = 3)

Samples	Added	Total found	Recovery	RSD
1	0	0. 95 pM		
2	2.50 pM	3.40 pM	98.0%	2.87%
3	10.0 pM	11.1 pM	102%	5.74%
4	250 pM	244 pM	97.2%	5.21%
5	1.00 nM	0.969 nM	96.8%	3.40%
6	25.0 nM	26.0 nM	104%	4.03%

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