Supporting Information

Electrochemiluminescence Biosensor for Kras Mutations Based on Locked Nucleic Acid Functionalized DNA Walkers and Hyperbranched Rolling Circle Amplification

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1. Materials and Instruments

1.1. Materials and reagents

Streptavidin magnesphere paramagnetic particles (PMPs) were purchased from Promega Corporation (Madison, USA). Amicon ultra-0.5 centrifugal filter devices (Amicon ultra 3 K device) were provided by Merck Millipore Ltd. (Darmstadt, Germany). Dichlorotris (1,10-phenanthroline) ruthenium(II) hydrate ($Ru(phen)_3^{2+}$), Tween-20, and tripropylamine (TPA) were purchased from Sigma-Aldrich (Shanghai, China). Escherichia coli (E. coli) DNA ligase set including E. coli DNA ligase, 10× E. coli DNA ligase buffer, and 10× BSA (0.05%) were obtained from Takara Biotechnology Co., Ltd. (Dalian, China). Exonuclease I (Exo I), Exonuclease III (Exo III), and their corresponding buffer were purchased from Thermo (Shanghai, China). Nicking endonuclease (Nb.BbvCI), Bst DNA polymerase large fragment, and their corresponding buffer were purchased from New England Biolabs (Beijing, China). SYBR Green I and SYBR Green II were provided by Xiamen Biovision Biotechnology Co., Ltd. (Xiamen, China). The deoxynucleotide solution mixture (dNTPs), agarose, and all oligonucleotides (Table S1) used in this work were obtained from Sangon Inc. (Shanghai, China). Tris-acetate-EDTA (50× TAE) and Tris-borate-EDTA (5× TBE) were purchased from Shanghai Double-helix Biotech Co., Ltd. (Shanghai, China).

Buffer solutions used in this project:

Buffer 1: 50 mM NaCl, 10 mM Tris-HCl, and 10 mM $MgCl_2$ (pH 7.9).

Buffer 2: 100 mM NaCl, 100 mM sodium phosphate buffer solution, 0.05% Tween-20 (pH 7.4).

Buffer 3: 100 mM NaCl, 100 mM sodium phosphate buffer solution (pH 7.4).

Buffer 4: 70 mM Tris-HCl, 10 mM $MgCl_2$ (pH 8.0).

All other chemicals were of analytical reagent grade and used directly without further purification. Double-distilled water (Milli-Q, Millipore, resistance 18.2 MΩ·cm) was used throughout the study.

1.2. Instruments

The ECL signals were recorded by the lab-made system including a CHI660D electrochemical workstation (Chenhua Instruments, Shanghai, China) and a BPCL ultra weak luminescence analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China). A gold (3 mm in diameter), platinum, and Ag/AgCl were used as working, counter and reference electrodes, respectively. The operating voltage of PMT was set as −800 V.

2. Construction and Characterization of the DNA Walkers

To structure the DNA walkers, biotin modified DNA walker tracks (DWs) and LNA modified protecting probe (LMPP), separately dissolved in buffer 1, were incubated at 90 °C for 10 min and hybridized by slowly cooling to room temperature to form protected DNA walkers probe (PWs). A portion of PMPs (1 mg/mL) was washed by buffer 2 three times and then dispersed in buffer 3 solution. The PWs and biotin modified substrate strands DNA (SDs) at a ratio of 1 to 20 were mixed, and then added to the PMPs solution with slight shaking for 60 min at ambient temperature. Because of the specific interaction between streptavidin and biotin, the biotin modified DNA were tightly bound with streptavidin PMPs to achieve a

mixing solution (SDs-PWs-PMPs) containing SDs oligonucleotides (1500 nM) and PWs oligonucleotides (75 nM).

For a typical DNA walkers process, a certain concentration of target allele, prepared SDs-PWs-PMPs (150 nM), Nb.BbvCI (10 U), and 1 × NEB CutSmart buffer were mixed. The DNA machine was carried out at 37 °C for 10 min and terminated at 80 °C for 20 min. The supernatant was separated from the mixture by a magnet for later HRCA project.

3. Hyperbranched Rolling Circle Amplification and ECL Detection

Initially, the dumbbell-shaped probes (DPs) were denatured at 90 °C for 10 min, and cooled down to room temperature for approx. 60 min to ensure the formation of dumbbell-shaped. Then, the ligation system including DPs, E. coli DNA ligase (60 U), 10 × E. coli DNA ligase buffer, and 10× BSA (0.05%) was done at 37 °C for 120 min and heated at 65 °C for 10 min to terminate the reaction. The addition of Exo I (10 U) and Exo III (20 U) were incubated at 37 °C for 120 min to digest the non-ligatured probe, the digestion reaction was ended by thermal treatment at 80 °C for 20 min. These prepared ligation DPs, used as template probe of HRCA to diminish nonspecific amplification and reduce the reaction time of HRCA, were stored at 4 °C for further use.

After the separation from the mixture by a magnet, the supernatant (50 μL) was used to trigger the HRCA reaction at 63 °C for 90 min in the solution containing 10 μL of prepared DPs, primer 1 (1 μM), and dNTPs (10 mM), 2 μL of 10 × BSA, 8 U Bst DNA polymerase large fragment and its 1× buffer. Then, the HRCA products were mixed

with 2 μL of 1 mM $Ru(phen)_3^{2+}$ for 180 min at ambient temperature to ensure the insertion of $Ru(phen)_3^{2+}$ into the groove of double strands DNA (dsDNA).[1] Besides, the Amicon ultra 3 K device was employed to filter the needless $Ru(phen)_3^{2+}$ molecules and DPs according to the instruction manual (the amicon was spun at 14,000 × g for 20 min). Then the concentrated sample was transferred from the device to a clean tube by spinning for 2 min at 1,000 × g.

The ECL determination liquid was consisted of resulting concentrated solution, phosphate buffer saline (200 mM, pH 7.4), and TPA (20 mM, acted as coreactant[2]). And the ECL signals were recorded from 0.6 to 1.6 V (potential) with 100 mV/s of scanning speed.

4. DetectionFluorescence Assay and Gel Electrophoresis

Fluorescence method and agarose gel electrophoresis were carried out to demonstrate the feasibility of the proposed biosensor (The fluorescence signals were recorded by a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Corporation, USA) at room temperature.). SYBR Green I was added to the HRCA products including different lengths of dsDNA, and then hatched at room temperature for 15 min. The emission spectrum was from 500 to 600 nm with the excitation wavelength of 488 nm. The excitation and emission slit widths were set as 10.0 nm.

As for gel electrophoresis, 20% polyacrylamide gel electrophoresis was employed to verify the establishment of DNA walkers in the 1× TBE buffer (pH 8.2), and 1.7% agarose gel electrophoresis was performed to demonstrate the dual signal

amplification approach in the 1× TAE (pH 8.0) buffer at a constant voltage of 80 V for 60 min at ambient temperature.

5. Cell Lysate Sample Analysis.

To remove the supernatant, the cells were centrifuged at 2000 rpm for 5 min at 4 °C, and the cells precipitate were resuspended in a cold Tris-HCl buffer (buffer 4). Then, the obtained solution was treated by ultrasonic cell disruption system (90 W) with 2 s on and 5 s off for 10 min. Finally, the prepared cellular homogenate samples (1.0×10^5 cells/mL) were stored at 4 °C for further use.

Figure S1

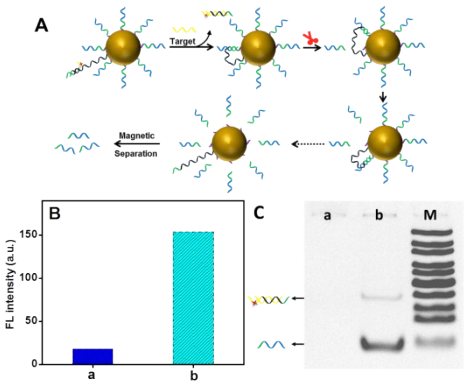

Fig.S1 (A) Schematic illustrating the mechanism of the DNA walkers. (B) Fluorescence analysis of movement results of the DNA walkers: (a) in the absence of target DNA, (b) in the presence of 50 fM target DNA. (C) Polyacrylamide gel electrophoresis analysis of movement results of the DNA walkers, the lanes a, b were corresponding with above, lane M indicated DNA size marker.

Initially, the movement of DNA walkers was tested by a fluorescence method (Fig. S1B). Only weak signal was detected without the addition of target DNA (curve a). However, upon the addition of target 134A sequence and Nb.BbvCI, many short probes DNA (SPs) were produced through the cleavage, which could combine with SYBR Green II and hence produce a strong fluorescence signal (curve b). The products were further analyzed by polyacrylamide gel electrophoresis. As shown in Fig. S1C, no band appeared without the addition of target DNA (lane a). But two obvious bands were observed if the target DNA was added, one was the hybridization product between target DNA with LMPP and the other one was the free SPs. These results indicate that (1) the DWs can be activated by the target DNA and (2) the DNA walkers are well established.

Figure S2

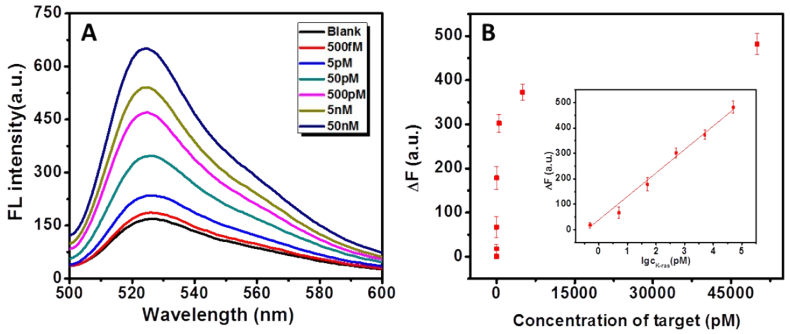

Fig.S2 (A) Fluorescence analysis at different concentrations of target Kras gene sequence. (B) The relationship between FL intensity and the Kras DNA sequence concentration. Inset figure: The calibration curve between the *Δ*F and the logarithm of the Kras DNA sequence concentration. The error bars represented the standard deviation of three replicate detections.

To further confirm the availability of the whole system, the resulting products of different concentration of target were detected by a fluorescent method via the mechanism that SYBR Green I can be embedded into dsDNA and thus yields strong fluorescence signal (as shown in Fig. S2). The fluorescence intensity enhanced as the target concentration rose. There is a linear relationship between the fluorescence intensity (ΔF, the difference value of fluoresce signal detected in the presence and absence of target) and the logarithm target ranging from 500 fM to 50nM. The regression equation is $\Delta F = 92.2 \lg c_{target} + 38.7$ (regression coefficient was 0.991). The detection limit was calculated to be 320 fM (calculated by the protocol of the definition of $3\sigma_b/s$, where σ_b is the standard deviation of the blank sample, and s is the slope). Apparently, compared to the fluorescence strategy, the detection for Kras mutation using the proposed ECL biosensor (LOD of 0.03 fM) is much more sensitive.

Table S1 DNA sequences and modifications

DNA name	Sequences
SDs	5′-biotin-TTTTTT-GC*TGA GGT AGT AGG TTG TAT AGT T-3′ (*cleavage site)
DWs	5′-biotin-T-40-T-T GGC GTA GGC AAG AGT GCC TCA GC-3′
LMPP	5′-TGAGG CAC TCT TGC CTA CGC CA CTA GCT CCA-3′ (T was modified by LNA)
DPs	5′-p-ACCTCATTGTATAGCCCCCCCCTGAGGTAGTAGGTTGCCCAACTATACAACCTACT-3′
Primer 1	5′-TGA GGT AGT AGG TTG TAT AGT T-3′
Target for 134A allele	5′-GTT GGA GCT AGT GGC GTA GGC AAG AGT G-3′
Wild type	5′-GTT GGA GCT GGT GGC GTA GGC AAG AGT G-3′
135A allele	5′-GTT GGA GCT GAT GGC GTA GGC AAG AGT G-3′
135C allele	5′-GTT GGA GCT GCT GGC GTA GGC AAG AGT G-3′
135T allele	5′-GTT GGA GCT GTT GGC GTA GGC AAG AGT G-3′
134C allele	5′-GTT GGA GCT CGT GGC GTA GGC AAG AGT G-3′
134T allele	5′-GTT GGA GCT TGT GGC GTA GGC AAG AGT G-3′
138A allele	5′-GTT GGA GCT GGT GAC GTA GGC AAG AGT G-3′

Table S2 Comparison between other strategies and the proposed ECL biosensor for Kras gene mutation assay

Method	Dynamic range	Detection limit
Fluorescent detection based on toehold-mediated strand displacement reaction[3]	0.002 to 10 nM	1.8 pM
Fluorescent Method based on double-hairpin molecular beacon[4]	0.05 to 200 nM	50 pM
Electrochemical clamp assay[5]	0.168 to 168 pM (1 fg/μL to 100 pg/μL)	168 fM (1 fg/μL)
Electrochemical biosensor based on functional composite nanofibers[6]	0.1 to 100 pM	30 fM
The proposed method	0.05 fM to 5pM	0.03 fM

Table S3 Detection of target 134A mutant in cellular lysate with the established ECL biosensor.

Sample		Target DNA added (fM)	Target DNA detected (fM)	Recovery rate (%)	RSD (%)
Cell lysate	1	0.10	0.11	110	8.8
	2	15.00	15.80	105	2.5
	3	100.00	109.28	109	5.7
	4	600.00	556.39	92.7	5.5

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