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Linlin Yang, Yingzhou Tao, Guiyin Yue, Ruibao Li, Bin Qiu, Longhua Guo,Zhenyu Lin * and Huang-Hao Yang

Ministry of Education Key Laboratory of Analysis and Detection for Food Safety, Fujian Provincial Key Laboratory of Analysis and Detection for Food Safety, Fuzhou University, Fuzhou, Fujian, 350116, China

Corresponding author: Zhenyu Lin
E-mail: zylin@fzu.edu.cn; Tel&Fax: 86-591-22866135

Address: Department of Chemistry, Fuzhou University, Fuzhou, Fujian, 350116, China

Abstract

An ultrasensitive and specific electrochemiluminescence (ECL) biosensor has been designed for p53 DNA sequence, which is based on cascade signal amplification of nicking endonuclease assisted target recycling and hyperbranched rolling circle amplification (HRCA). First of all, biotin modified hairpin capture DNA (HP) probe was immobilized on the surface of streptavidin magnespheres paramagnetic particles (PMPs). Target DNA hybridized with the loop portion of the HP probe, therefore unfolding HP to form a double-stranded DNA (dsDNA) containing the specific nicking site of the nicking endonuclease. Then the nicking endonuclease recognized the specific nicking site and cleaved the HP into two pieces, liberating target DNA and the complementary sequence piece for the padlock probe. The intact target DNA would initiate the next cycle of hybridization and cleavage, thereby releasing multiple complementary sequences for the padlock probes. The liberated complementary sequences hybridized with the padlock probes, subsequently inducing the HRCA reaction and generating numerous dsDNA segments. Herein, $Ru(phen)_3^{2+}$ which has embedded into dsDNA and worked as ECL signal reporter. The reaction products were eventually pretreated by dialysis tube with the cut-off membrane to remove the residual $Ru(phen)_3^{2+}$ in the solution for the following ECL measurements. Using this cascade amplification strategy, an ultrasensitive p53 DNA sequence detection method was developed with a wide linear ranging from 0.05 fM to 100 fM and a low detection limit of 0.02 fM. Moreover, this cascade amplified ECL biosensor had

specific recognition capacity for non-complementary, single- and double-base mismatched DNA. The proposed ECL biosensor might have a great potential in the biomedical research and clinic analysis.

The p53 gene is a kind of tumor suppressor gene and plays a crucial role in the regulation of the cell cycle, apoptosis, cell differentiation, DNA repair and other biological functions.[1] The p53 tumor suppressor gene can code and express p53 protein which can suppress cell malignant transformation, so p53 gene has a general antitumor effect and is known as "the guardian of genome".[2] About 50% of all malignant tumors have found mutant p53 gene, mutations in the p53 gene have been the most common genetic alteration in human cancers.[3-5] Therefore, sensitive and rapid detection of p53 gene plays important roles in early screening of cancers. Various measuring techniques including electrochemistry,[6,7] fluorescence,[8,9] chemiluminescence,[10] electrochemiluminescence (ECL), [11,12] and colorimeter[13] have been implemented to achieve sensitive detection of p53 DNA sequence. Among these techniques, ECL has attracted particular attention and is becoming more recognized for DNA detection due to its high sensitivity, rapidness, simple equipment and low cost.[14]

In order to improve the detection sensitivity and realize the trace level detection, a series of powerful amplification strategies such as nanomaterial-based assays,[15] nuclease-assisted target recycling strategies,[16,17] polymerase-assisted amplification,[18,19] and hybridization-based technologies[20] have been widely applied for nucleic acid analysis. Specifically, nicking endonuclease signal amplification (NESA) has received increasing concern because of its intrinsic ability for extremely specific recognition.[21-24] Nt.BstNBI, one kind of nicking endonucleases, owns distinct

selectivity for specific DNA sequences such as 5′-GAGTC-3′ in dsDNA, it initially recognizes the site and then only cleaves the 4th base from its 3′ end.[25] Our early study proved that NESA had distinct specificity for the detection of p53 DNA sequence and could even distinguish a single-base mismatched oligonucleotide.[13] However the sensitivity of such method should be further improved (only in picomolar level). Polymerase chain reaction (PCR) has been developed as a mature and widely used technology in the amplified assay of genes,[26,27] whereas it is often limited by the drawbacks of temperature programmer, vulnerability to contamination and sophisticated equipment.[28,29] Rolling circle amplification (RCA), by contrast, is an isothermal enzymatic DNA replication process, this process is simpler and more convenient than PCR. But the amplification efficiency of RCA is limited, it is less sensitive than that of PCR.[30] Hyperbranched rolling circle amplification (HRCA) evolved from RCA can be achieved under isothermal environment, but it has much higher amplification efficiency (10^9-fold) and can generate numerous varying length dsDNA segments.[31] In our early study, different detection techniques had been coupled with HRCA to develop sensitive biosensors.[32-35] However, the recognition capacity against a single-base mismatch of HRCA-based biosensor still remains challenging because the replication steps may introduce false positive results from non-specific amplification.

Herein, we firstly proposed a cascade amplified ECL biosensor which combined the superior recognition capacity of NESA towards the single-base mismatched DNA

and drastic signal amplification efficiency of HRCA for highly selective and sensitive detection of p53 DNA sequence. As the ECL signal reporter, $Ru(phen)_3^{2+}$ which had embedded into dsDNA of the HRCA products.[36] Moreover, magnetic bead, a versatile tool frequently used in the separation of nucleic acids, proteins and other biomacromolecules,[37] was employed to separate the unreacted hairpin capture DNA probes from the solution and consequently further decreasing the background signal. The proposed ECL biosensor had high sensitivity and specificity for p53 DNA sequence. This cascade signal amplification strategy also provides a versatile tool for detecting nucleic acid in bioanalysis and clinical biomedicine.

Experimental Section

Reagents. Amicon Ultra-0.5 Centrifugal Filter Devices (Amicon Ultra 3 K device) was purchased from Merck Millipore Ltd. (Darmstadt, Germany). Streptavidin magnespheres paramagnetic particles (PMPs) were purchased from Promega Corporation (Madison, USA). Dichlorotris (1,10-phenanthroline) ruthenium(II) hydrate ($Ru(phen)_3^{2+}$), tween-20, bovine serum albumin (BSA) and tripropylamine (TPA) were obtained from Sigma-Aldrich (St. Louis, MO), they could directly utilize without additional purifying. SYBR Green I (10000×) was purchased from Xiamen Biovision Biotechnology Co. Ltd. (Xiamen, China). The nicking endonuclease (Nt.BstNBI), T4 DNA ligase and Bst2.0 DNA Polymerase were purchased from New England Biolabs (Beijing, China). Deoxynucleotide solution mixture (dNTPs),

proteinase K solution, agarose, adenosine triphosphate (ATP) and all involved sequences (listed as below) were obtained from Sangon (Shanghai, China).

Target DNA (p53 DNA sequence): 5′-TCA TCA CAC TGG AAG ACT C-3′

Hairpin capture DNA (HP): 5′-Biotin-TTT TTT *TCC CGA TCC* **GAG TCT TCC**↓ **AGT GTG ATG A**AC T*GG ATC GGG A*-3′ (the italic parts at the two ends represented the complementary sequences of the stem arm and the bold part was complementary to the target DNA; the dotted and underlined sections corresponded to the complementary region for padlock probe and the Nt.BstNBI recognition oligonucleotides, respectively; the right arrow indicated the nicking site,).

Padlock probe: 5′-P-GTT CAT CAC ACT TAT CCT TTG GTT GAA ACT TCT TCC TTT CTT TCC CGA TCC A-3′ (the dashed part matched the hairpin probe while those marked by wavy line and underlined referred to the binding portions for Primer 1 and 2, respectively).

Primer 1: 5′-TTC AAC CAA AGG ATA-3′

Primer 2: 5′-ACT TCT TCC TTT CTT-3′

Single-base mismatched DNA (T1): 5′-TCA TCA CAC TGG AAG AAT C-3′,

Double-base mismatched DNA (T2): 5′-TCA TCA CAC TGG AAG GAT C-3′

Non-complementary DNA (Tn): 5′-GGT CTC TTG ATA GCA CTC A-3′

All other used materials were analytical grade reagents and DI water (Milli-Q, 18.2 MΩ) was utilized in all experiments. Buffer solution used in this study:

Buffer A: 0.1 M NaCl, 0.1 M sodium phosphate buffer solution, pH 7.4, 0.05% Tween-20.

Buffer B: 0.1 M NaCl, 0.1 M sodium phosphate buffer solution, pH 7.4.

NEBuffer 3: 100 mM NaCl, 50 mM Tris-HCl, 1 mM dithiothreitol and 10 mM $MgCl_2$, pH 7.9.

Instruments. Signals that were plotted based on ECL intensity against potential were obtained 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). -900 V was the operating voltage in the photomultiplier tube (PMT) of the latter instrument. Meanwhile, a traditional three-electrode system was employed for all experiments, in which platinum wire, gold (3 mm in diameter) and Ag/AgCl (saturated with KCl) served as counter, working and reference electrodes, respectively.

Immobilization of Biotin Modified HP Probe onto Streptavidin Magnespheres Paramagnetic Particles. Biotin modified HP probe was annealed at 90 ℃ for 5 min and followed by slowly decreasing to room temperature. A portion of 0.6 mL of 1 mg/mL PMPs was washed three times by buffer A solution and then dispersed in buffer B solution. Then biotin modified HP probe was added into the solution to achieve a final concentration of 1 μM, and the mixture was placed on a shaker for 1 h at room temperature. As a result, PMPs with HP (HP-PMPs) were formed due to the specific combination of streptavidin with biotin and can be separated from the mixture

by a magnet for the paramagnetic property of PMPs. Subsequently, HP-PMPs were washed by buffer A solution for three times and resuspended in buffer B solution. Finally, they were stored at 4 ℃ for the later use.

Nicking Endonuclease Signal Amplification. Target DNA with varying concentrations and 2.5 μL of the obtained HP-PMPs were incubated in the 50 μL of 1× NEBuffer 3 at 37 ℃ for 2 h. After that, 6 U Nt.BstNBI were added. The recycling process was performed at 55 ℃ for 1 h. At last, the HP-PMPs was separated from the mixture by a magnet, and supernatant was remained for later experiments.

Hyperbranched Rolling Circle Amplification. 2.5 μL of 1 μM padlock probe was firstly added to the achieved supernatant and incubated at 37 ℃ for 1 h. Secondly, the ligation reaction occurred in the mixture containing 50 μL of 1× T4 DNA Ligase buffer, 10 U T4 DNA Ligase and 1 mM ATP at 37 ℃ within 1 h. Thirdly, the HRCA-reaction occurred at 63 ℃ within 1 h via addition of 100 μL of 1× Isothermal Amplification Buffer, 8 U Bst2.0 DNA Polymerase, 2.5 μL of 1 μM primer 1, 2.5 μL of 1 μM primer 2, and 20 μL of 10 mM dNTPs. Fourthly, 2 μL of 1 mM $Ru(phen)_3^{2+}$ was added to HRCA reaction products and incubated at room temperature for 3 h to make $Ru(phen)_3^{2+}$ molecules embed into dsDNA. Then the reaction products were added to the Amicon Ultra 3 K device, and treated following the instruction manual (the device was spun at 14000 × g for 20 min at room temperature). Finally, the

concentrated $Ru(phen)_3^{2+}$ molecules which have embedded into dsDNA were transferred immediately by reverse spin of the filter (1000 × g for 2 min).

ECL Determination. The resulting ECL intensity was determined from 0.6 to 1.6 V (potential) and the scanning speed was 100 mV/s. It was achieved in phosphate buffer (0.2 M, pH 7.4) consisting of TPA (0.02 M). Reference result (average of three times, indicated by error bar) was eventually calculated.

Gel Electrophoresis and Fluorescence Measurement. 2% agarose gel electrophoresis and the fluorescence measurement were carried out to verify the cascade signal amplification. The electrophoresis was carried out in 0.5× Tris-borate-EDTA (TBE) (pH 8.0) at a constant voltage of 80 V for 1 h at room temperature. After staining by EB solution for 15 min, gels were photographed by gel image system. As for the fluorescence measurement, the products of the cascade amplification reaction were mixed with SYBR Green I (ultimate concentration of 1×) and then incubated at room temperature for 15 min. The fluorescence emission spectra were collected from 510 to 590 nm with the excitation wavelength of 488 nm.

Preparation and DNA Assay in Human Serum and Cellular Homogenate. Human serum samples from volunteers were pre-incubated with proteinase K (400 μg/ml) at 37 ℃ for 1 h and then diluted with 1× NEBuffer 3. The cellular homogenate was prepared according to our previous research[38]. Briefly, the normal liver cells (1.0×10^6 cells) were centrifuged for 5 min at 25 ℃ (1000 rpm) to remove the supernatant, and the cells precipitate was resuspended in 1.0 mL of 1× NEBuffer 3. Then, the above

solution was subjected to a sonication treatment for 30 min (with 4 s on and 8 s off) in an ice-water bath using a probe-type sonicator (200 W), and the cellular homogenate was used immediately or stored at 4 ℃. Finally, p53 DNA sequences were added in diluted human serum (10%) and cellular homogenate samples (1.0×10^5 cells/mL) separately, and detected according to the above procedure.

Results and Discussions

The Mechanism regarding the Proposed Biosensor. Scheme 1 systematically presented the mechanism regarding this proposed cascade signal amplification ECL biosensor. The biotin modified HP probe was initially bonded to PMPs surface via the specific combination of biotin with streptavidin. Once the target DNA was introduced into the solution, the target DNA hybridized with the loop portion of the HP probe, hence unfolding HP to form a duplex conformation. Nicking endonuclease Nt.BstNBI specifically recognized the specific asymmetric sequence 5′-GAGTC-3′ in this duplex, resulting in cleaving the 4^{th} base from its 3′ end. In this way, the HP probe strand was cleaved into two pieces and the complementary sequence piece for the 5′-phosphorylated linear padlock probe was released from PMPs surface to hybridize with the padlock probe. In the meantime, the intact target DNA also spontaneously dissociated from the immobilized piece on the PMPs surface because the unwinding temperature (44.7 ℃) was below the nicking reaction temperature (55 ℃). Furthermore, it hybridized with a new HP probe and initiated the next cycle of

cleavage, leading to a target DNA recycling process and the production of multiple complementary sequences for the padlock probes. After this Nt.BstNBI-assisted target recycling process, the residual HP-PMPs were separated out by a magnet. Subsequently, the padlock probe was introduced and the dashed part of the padlock probe hybridized with the released complementary sequence. Ligation and circularization of the padlock probe were completed by T4 DNA Ligase treatment following. Afterwards, the HRCA process was initiated by Bst2.0 DNA Polymerase. In brief, the Primer 1 firstly hybridized with the wavy line region of the resulting circular padlock probe. Upon adding Bst2.0 DNA Polymerase, such Primer 1 was stretched at its 3′ end under isothermal environment and thus yielding single-stranded DNA (ssDNA). The obtained ssDNA contained multiple complementary sequences to padlock probe and one portion of padlock probe was same as Prime 2, so Primer 2 could hybridize with the extension product of Primer 1 and then was further stretched to replace the previously extended DNA under the same conditions. Such replaced strand possessing many binding sites for Primer 1 was available for hybridization again, thereby generating numerous varying length dsDNA segments.[32,33] Afterwards, $Ru(phen)_3^{2+}$ which had embedded into dsDNA was added and then the reaction products were pretreated by dialysis tube with the cut-off membrane to remove the residual $Ru(phen)_3^{2+}$ molecules for the following ECL measurements. The number of target DNA was positively related to the enhanced ECL intensity. Specifically, the HP probe could not be cleaved by Nt.BstNBI without target DNA. Therefore, the target

DNA recycling process could not be initiated to release complementary sequences for padlock probes, so no HRCA reaction was triggered and only a weak ECL signal was detected.

To evaluate the feasibility of the cascade signal amplification ECL biosensor, the electrochemical and ECL signals in the presence and absence of target DNA, Nt.BstNBI and Bst2.0 DNA Polymerase were recorded. Curve a in Figure 1A (the inset figure is the corresponding CV curves) showed that ECL signal and anodic current of $Ru(phen)_3^{2+}$ oxidation were both weak in the presence of Nt.BstNBI and Bst2.0 DNA Polymerase but without target DNA, indicating that the number of dsDNA segments regarding the cascade amplification reaction was few. Whereas obvious anodic current and ECL signal enhancement appeared (Curve b) when target DNA, Nt.BstNBI and Bst2.0 DNA Polymerase were all presented. In the presence of target DNA and Bst2.0 DNA Polymerase but without Nt.BstNBI (Curve c), or in the presence of target DNA and Nt.BstNBI but without Bst2.0 DNA Polymerase (Curve d), there was little change in both ECL signal and anodic current as compared with Curve a, indicating that few dsDNA was produced and no cascade amplification reaction occurred. These phenomena indicated that: (1) the cascade amplification reaction happened; (2) a large quantity of dsDNA were formed; and (3) $Ru(phen)_3^{2+}$ which had embedded into dsDNA was the ECL signal reporter. According to the above experiment, the cascade amplification reaction did not occur in the absence of target DNA. However, when Nt.BstNBI-assisted target recycling process was

performed in homogeneous solution instead of on the PMPs under the same conditions, a much higher background signal was observed (Curve e). That was because the intact HP probes could not be separated from the system without PMPs and some of them also could hybridize with the padlock probes to initiate HRCA reaction. These results clearly suggested that the employment of PMPs could cause low detection background and further improve the detection sensitivity.

To further prove the validity of the proposed cascade signal amplification process, the resulting products were analyzed by performing gel electrophoresis experiments. As shown in Figure 1B (the gel electrophoresis image), no bands were observed when only two reactants were present (lane a, c and d), suggesting that no cascade amplification reaction occurred. Upon the introduction of all three reactants, an obvious smear appeared (lane b) because the molecular weight of resulting products was an approximate range, indicating numerous varying length dsDNA segments were yielded. The fluorescence of the system in the absence and presence of target DNA was applied to further verify the validity of the proposed system. Weak fluorescence signal appeared in the absence of target DNA (Curve a in Figure 1C), whereas an obvious fluorescence signal was detected in the presence of target DNA (Curve b in Figure 1C). These phenomena verified that target DNA could initiate NESA and HRCA reaction and also confirmed the credibility of this proposed ECL biosensor.

Optimization of the Reaction Conditions. To achieve the best sensing performance, some parameters were optimized. The cleavage time and the HRCA reaction time were firstly optimized because they directly affected the quantity of dsDNA. Figure 2A revealed that the ECL intensity rose significantly with increasing the cleavage time initially and eventually reached a plateau after 1 h, indicating that 60 min was enough to complete the cleavage. As for the followed HRCA reaction time, the ECL intensity raised distinctly with enhancing the HRCA reaction time from 0 to 60 min, but remained unchanged between 60 and 100 min. ECL signals of the cleavage and HRCA reaction both reached a plateau at 60 min, which could be explained by substrate depletion or production inhibition. Thus 60 min was chosen as the optimal time for the cleavage and HRCA reaction.

The effect of the spin time of dialysis on the ECL intensity of the system was also investigated because the presence of the free $Ru(phen)_3^{2+}$ which has not embedded into dsDNA can cause high background signal. As shown in Figure 2B (red curve), the ECL intensity decreased remarkably with raising the spin time firstly and then reached a plateau at 20 min. This suggested that the free $Ru(phen)_3^{2+}$ was removed thoroughly, so 20 min was chosen for the following study. Additional, Figure 2B (black curve) illustrated the relationship between the ECL intensity and incubation time for embedding $Ru(phen)_3^{2+}$ into dsDNA. Two trends were involved for ECL signal: (1) it rose slightly with raising the incubation time from 1 to 3 h; and (2) it

achieved at its plateau over 3 h. Consequently, the optimized incubation time was 3 h in this assay.

Nt.BstNBI and Bst2.0 DNA Polymerase involved in this system were crucial important for target DNA recycling process and HRCA process, respectively，so the effects of dosages of Nt.BstNBI and Bst2.0 DNA Polymerase were investigated as well. Figure 2C showed that the ECL intensity enhanced rapidly until 6 U with increasing dosage of Nt.BstNBI and there was little change in the ECL signals between 6 and 12 U, indicating 6 U Nt.BstNBI was sufficient for cleavage and target DNA recycling. As for Bst2.0 DNA Polymerase, the ECL intensity enhanced evidently with the augmentation of Bst2.0 DNA Polymerase, and the maximum ECL intensity was obtained at 8 U. Increasing dosage of Bst2.0 DNA Polymerase from 3.2 to 8 U resulted in more HRCA products, but the ECL signal did not further increase beyond 8 U. Thus the optimized dosages of 6 U Nt.BstNBI and 8 U Bst2.0 DNA Polymerase were employed in this study. Figure 2D revealed that the quantity of dNTPs supplied for HRCA affected greatly on the sensitivity of the ECL biosensor. Specifically, the ECL intensity rose obviously with enhancing the concentration of dNTPs owing to the raising of HRCA products, and reached the plateau at 0.6 mM. This was probably because of the spatial steric effect and limited space. Consequently, 0.6 mM dNTPs was used in this assay.

Calibration Curve for Target DNA Determination. Under the optimized conditions, the ECL biosensor was used for the quantification of target DNA by adding different

concentrations of target DNA into the reaction system. Figure 3A showed the variation of the ECL intensity with the concentration of target DNA, ECL peak intensity increased with increasing target DNA concentration. Figure 3B plotted the increased ECL intensity (ΔECL, the intensity variation with and without target) and concentration of target DNA. ΔECL exhibited excellent linear relation with the logarithm of target DNA ranging from 0.05 fM to 100 fM. The regression equation was ΔECL/ counts = 2401.7 + 1027.9 lgC_{p53} (correlation coefficient was 0.9940). The detection limit was calculated to be 0.02 fM by evaluating the average signal of the blank plus three times the standard deviation. The sensitivity of this cascade signal amplification method was superior to that of DNAzyme and restriction endonuclease-assisted electrochemical method,[6] cascade DNA nanomachine-based signal amplification strategy,[8] isothermal polymerization-based amplification system,[9] nanomaterial-based electrochemical method,[7] and dual amplified electrochemical sensor based on Nt.BstNBI-assisted target recycling and RCA[39]. Apparently, the proposed ECL biosensor had desirable linear range and high sensitivity because of the cascade signal amplification.

Selectivity and Reproducibility of the Proposed Biosensor. The specificity was assessed by the control hybridization experiments for different DNA sequences including target DNA, non-complementary DNA Tn, single-base mismatched DNA T1 and double-base mismatched DNA T2. The concentrations of Tn, T1 and T2 were 100 times as many as that of target DNA (5 fM). As shown in Figure 4, ECL signals

of T1, T2 and Tn were similar to that of blank, so interfering signals were negligible. These results demonstrated the high specificity of the proposed biosensor. The reason might be that the specific cleavage site for Nt.BstNBI required a completely matched target and the incompletely matched target could not form the recognition site. From the practical point of view, reproducibility was another essential element for p53 DNA sequence quantification. The relative standard deviations (RSDs) of intra- and inter-assays (n = 3) were less than 5% when the target concentration was fixed at 5 fM, indicating that this amplification strategy owned superior precision and reproducibility.

Determination of p53 DNA Sequence in Complex Biological Matrices. To further investigate the feasibility and capability of the application of the proposed ECL biosensor, p53 DNA sequences were spiked in diluted human serum (10%) and normal liver cellular homogenate samples (1.0×10^5 cells/mL) separately. As shown in Table 1, the recovery ranged from 94.0 to 110%, and RSDs were from 4.6 to 8.7%. Such obtained results enabled the proposed ECL biosensor to be applied more effectively and reliably in biomedical research and clinic analysis.

Conclusion

In summary, a highly sensitive and selective ECL approach based on cascade signal amplification was developed for p53 DNA sequence determination. This cascade signal amplification strategy exhibited high specificity and sensitivity owing to the

combination of superior distinction ability and amplification efficiency of Nt.BstNBI-assisted target recycling, drastic signal amplification power of HRCA as well as high sensitivity of ECL technique. The ECL biosensor was then applied to detect p53 DNA sequence in the human serum and cellular homogenate samples with satisfied results. Furthermore, with outstanding reproducibility and selectivity, the proposed biosensor was also employed to determine varying DNA sequences related to the specific recognition site.

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Figures and Captions

Scheme 1. The principle of cascade signal amplification based ECL biosensor for p53.

Figure 1. (A) ECL analysis of the amplification products: In the presence of Nt.BstNBI, Bst2.0 DNA Polymerase and PMPs, but without target DNA (a), in the presence of target DNA, Nt.BstNBI, Bst2.0 DNA Polymerase and PMPs (b), in the presence of target DNA, Bst2.0 DNA Polymerase and PMPs, but without Nt.BstNBI (c), in the presence of target DNA, Nt.BstNBI and PMPs, but without Bst2.0 DNA Polymerase (d), in the presence of Nt.BstNBI and Bst2.0 DNA Polymerase but without target DNA and PMPs (e) (the inset figure is the corresponding CV curves); (B) Agarose gel (2%) electrophoresis analysis of the amplification products, the experimental conditions for lanes a – d were indicated above, Lane M: DNA size marker; (C) Fluorescence measurement of the amplification products: In the absence of target DNA (a), in the presence of target DNA (b). 100 fM target DNA, 6 U Nt.BstNBI, and 8 U Bst2.0 DNA Polymerase were used in the experiments.

Figure 2. Optimization of the reaction conditions with 0.1 fM p53 DNA sequence: (A) Effect of the cleavage time and HRCA reaction time on the ECL intensity; (B) Effect of spin time of dialysis on the ECL intensity and effect of incubation time between $Ru(phen)_3^{2+}$ and dsDNA on the ECL intensity; (C) Effect of the dosage of

Nt.BstNBI and Bst2.0 DNA Polymerase on the ECL intensity; (D) Effect of dNTPs concentration on ECL intensity.

Figure 3. (A) ECL intensity at different p53 DNA sequence concentrations. a to i: 0, 0.05 fM, 0.1 fM, 0.5 fM, 1 fM, 5 fM, 10 fM, 50 fM, 100 fM; (B) The calibration curve between the logarithm of p53 DNA sequence concentrations and ΔECL. The error bars show the standard deviation of three replicate determinations.

Figure 4. The selectivity of the developed ECL biosensor. The error bars show the standard deviation of three replicate determinations.

Scheme 1

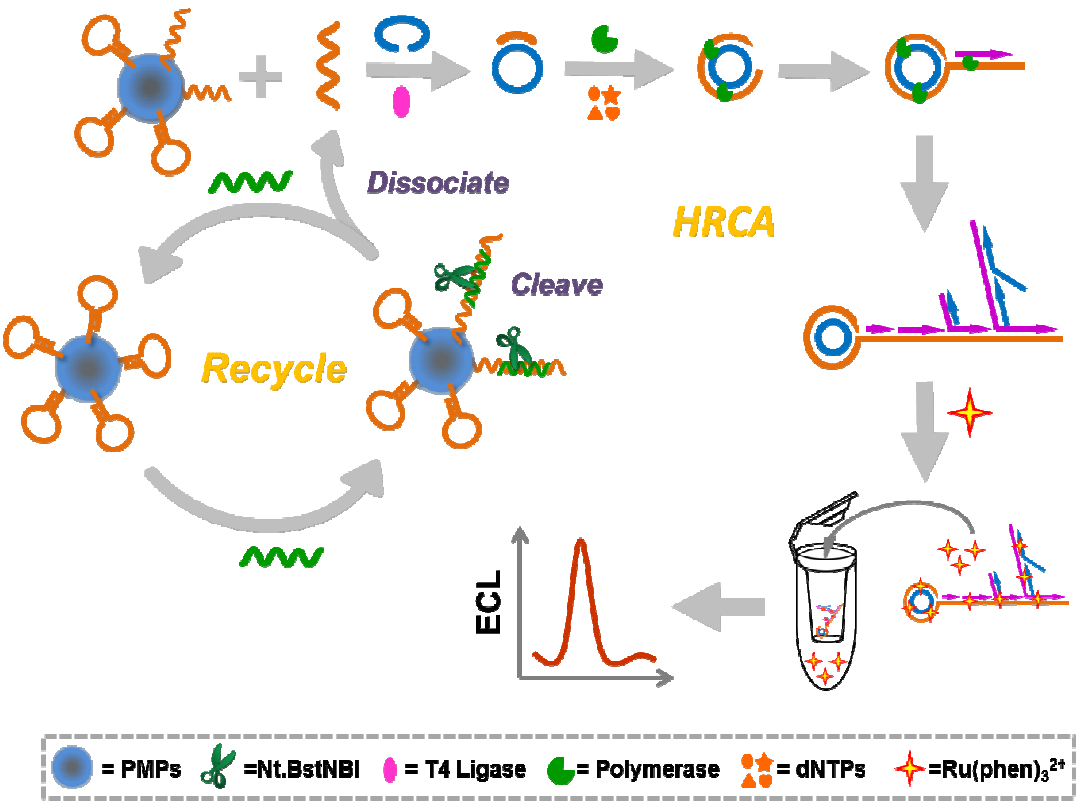
Dissociate
HRCA
Cleave
Recycle
ECL
= PMPs
=Nt.BstNBI
= T4 Ligase
= Polymerase
= dNTPs
=Ru(phen)3 2+

Figure 1

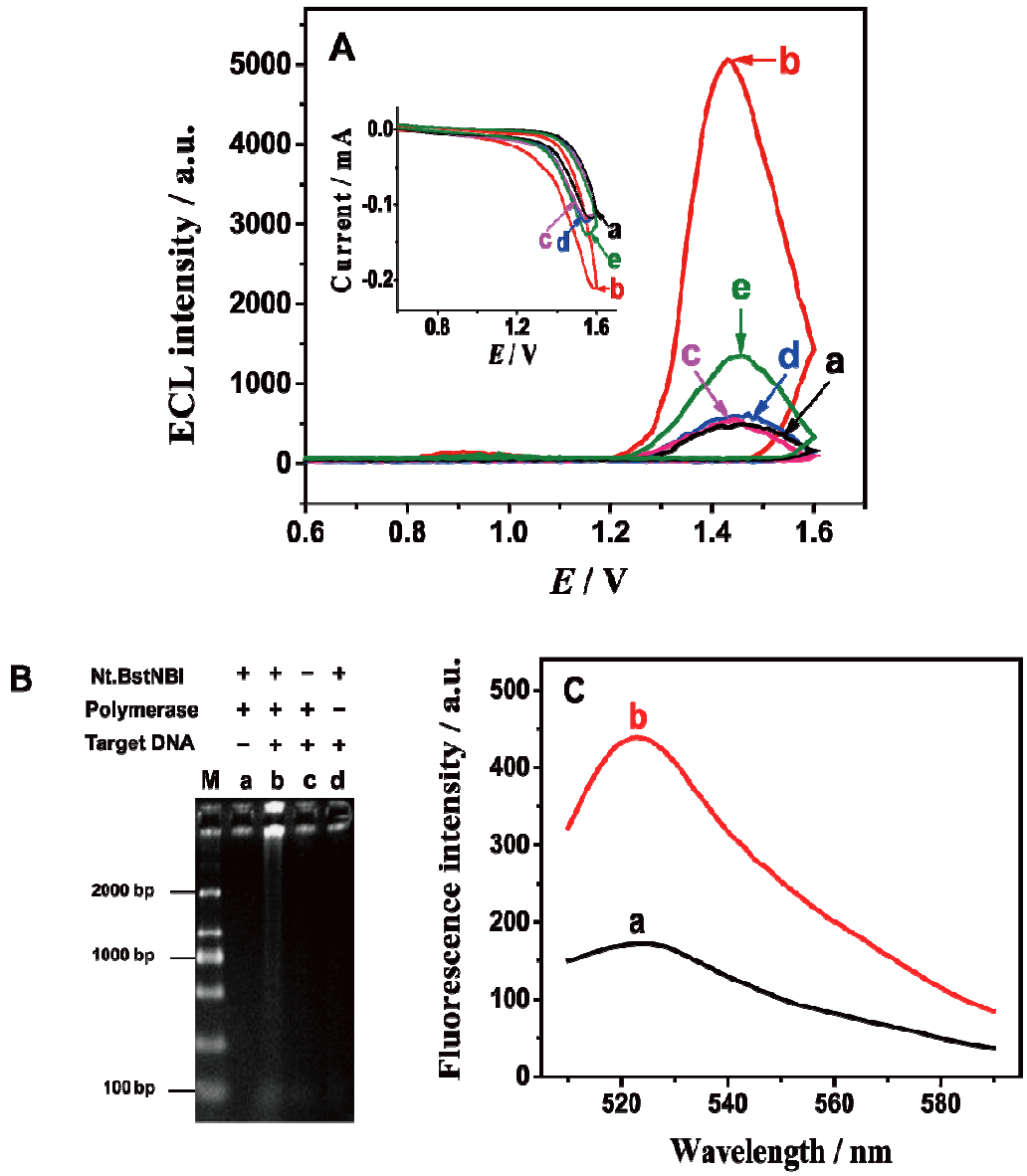
A
ECL intensity / a.u.
E / V
Current / mA
B
Nt.BstNBI
Polymerase
Target DNA
M a b c d
2000 bp
1000 bp
100 bp
C
Fluorescence intensity / a.u.
Wavelength / nm

Figure 2

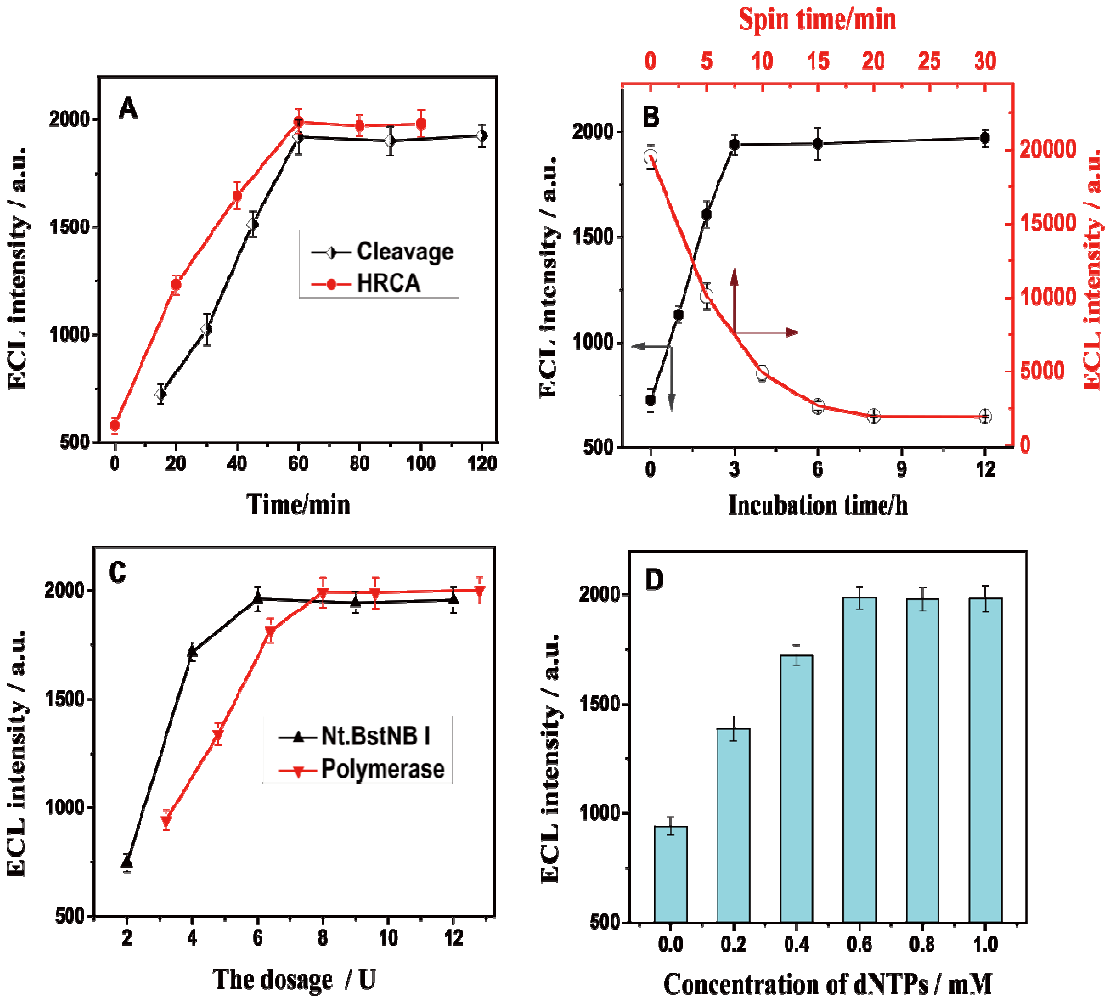
A
ECL intensity / a.u.
Time/min
Cleavage
HRCA
B
Spin time/min
ECL intensity / a.u.
Incubation time/h
C
ECL intensity / a.u.
The dosage / U
Nt.BstNB I
Polymerase
D
ECL intensity / a.u.
Concentration of dNTPs / mM

Figure 3

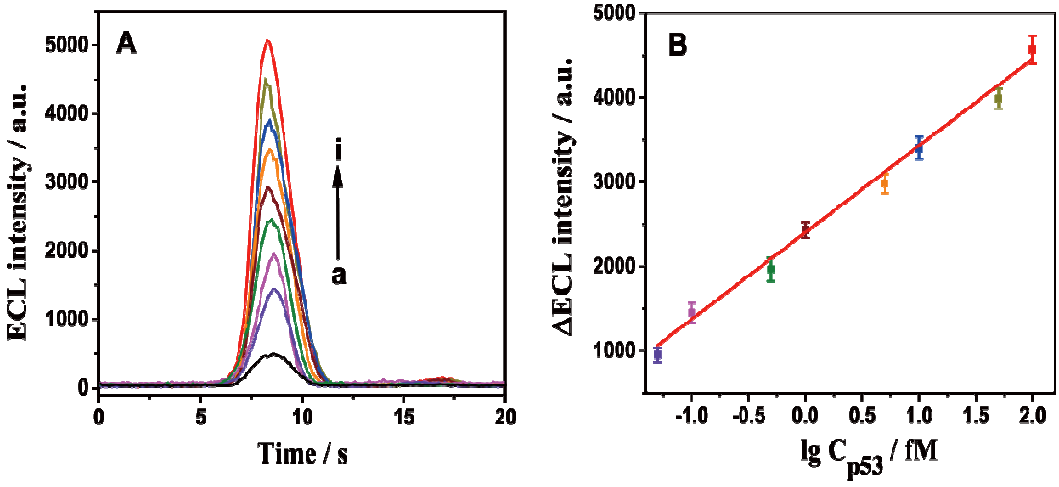

Figure 4

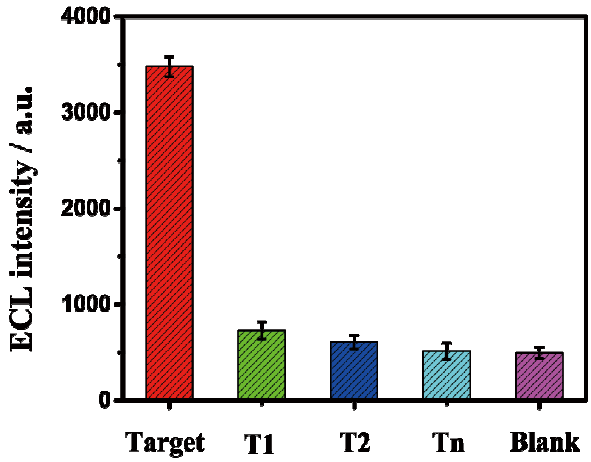

Table 1 Detection of p53 DNA sequence spiked in samples of human serum and cellular homogenate by the proposed ECL biosensor (n = 5)

Sample Name		Target DNA spiked (fM)	Target DNA detected (fM)	Recovery (%)	RSD (%)
Human serum	1	0.10	0.11	110	7.9
	2	1.00	0.94	94.0	4.6
	3	10.00	9.73	97.3	5.2
	4	50.00	49.26	98.5	6.1
Cellular homogenate	1	0.10	0.11	110	8.7
	2	1.00	1.04	104	7.4
	3	10.00	9.52	95.2	5.0
	4	50.00	48.93	97.9	8.3

For TOC only: