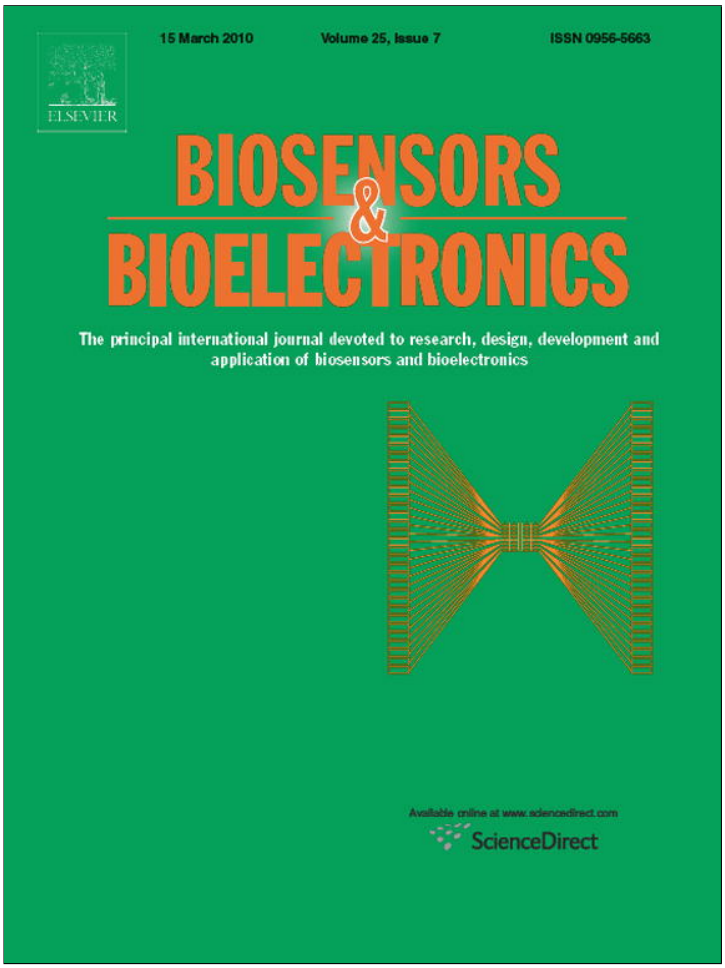

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Magnetic beads based rolling circle amplification–electrochemiluminescence assay for highly sensitive detection of point mutation

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ABSTRACT

The identification of point mutations is particularly essential in the fields of medical diagnosis and prognosis of many pathogenic and genetic diseases. In this study, an rolling circle amplification (RCA) based electrochemiluminescence (ECL) assay for highly sensitive point mutation detection was developed. In the assay, an allele-discriminating padlock probe was designed for targeting the sequence in the p53 oncogene locus. A circular template generated by enzymatic ligation upon the recognition of a point mutation (CGT to CAT) on the oncogene could be amplified isothermally by Phi29 DNA polymerase. The elongated products, containing hundreds of copies of the circular DNA template sequence, were hybridized with $Ru(bpy)_3^{2+}$ (TBR)-tagged probes and then captured onto streptavidin-coated paramagnetic beads. The resulting products were analyzed by magnetic bead based ECL platform. As low as 2 amol of mutated strands was detected by this assay, which could be attributed to the high amplification efficiency of Phi29 DNA polymerase and current magnetic bead based ECL detection platform. In addition, the positive mutation detection was achieved with a wild-type to mutant ratio of 10 000:1, due to the high fidelity of DNA ligase in differentiating mismatched bases at the ligation site. It is demonstrated that this proposed method provides a highly sensitive and specific approach for point mutation detection.

1. Introduction

It is commonly believed that human disease and genetic disorder are associated with the most common point mutations in human genome, thus the point mutation analysis plays an increasingly important role in medical diagnosis and prognosis of genetic-based diseases (McCarthy and Hilfiker, 2000; Baroni et al., 2004; Saal et al., 2008). The fact that cancer development and progression have a very strong association with the point mutation has been widely accepted (Pharoah et al., 2004; Whibley et al., 2009). Since the number and location of mutation should be evaluated to obtain an accurate diagnosis and prognosis of the disease, a reliable and highly sensitive DNA diagnostic method is urgently desired for differentiating allele mutation.

Up to now, a number of methods and technologies have been reported in the literature for efficient point mutation detection, including primer extension (Sokolov, 1990), oligonucleotide ligation (Landegren et al., 1988), enzymatic cleavage (Botstein et al., 1980; Lyamichev et al., 1999), allele-specific polymerase chain reaction (PCR) (Hacia et al., 1998), and TaqMan PCR (Fujii et al., 2000). Methods based on PCR dominate the field of allelic discrimination analysis due to its ability for the amplification of minute amounts of initial target sequences. However, PCR is considered to be complicated for a diagnostic setting as it requires sophisticated and expensive equipment and skilled operators to perform the assay. Moreover, it may introduce false positive results from non-specific amplification because of the complicated thermal cycling steps.

Recent developments in biotechnologies offer a wide variety of choices for nucleic acid amplification, among which rolling circle amplification (RCA) is a representative amplification technology (Nilsson et al., 1994, 2000; Lizardi et al., 1998; Hardenbol et al., 2003; Baner et al., 2003). In typical RCA, a circular template can be synthesized from a linear padlock probe, and the resulting circular template can be amplified with DNA polymerase using a primer complementary with the central region of the padlock probe. The produced single-strand DNA sequence contains thousands of repeated segments which are complementary with the circular template (Soengas et al., 1995; Nie et al., 2005). RCA has attracted considerable attention due to its high sensitivity obtained from powerful amplification under isotherm condition. So far, the RCA has found numerous applications in the detection of DNA (Li et al., 2008a,b; Guo et al., 2009; Zhang et al., 2009), proteins (Huang et al., 2007) as well as RNA (Zhang et al., 2008). Currently, electrophoresis and fluorescence based methods are dominantly available detection technologies for RCA. However, electrophoresis is generally considered as inefficient tool due to its laborious characteristic. As for fluorescence assays, the RCA products can be

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readily detected by hybridizing with fluorescent DNA probes. Nevertheless, immobilization of the amplification primer or the RCA products onto a solid support (gel, glass slides or chip) is complex and laborious, and the inefficient washing of excessive fluorescent probes will lead to the high fluorescence background inevitably. For improvements in the fluorescence based assays, real-time fluorescence monitoring has been developed by employing a molecular beacon design (Nilsson et al., 2002; Dahl et al., 2004). Furthermore, label-free fluorescence detection of RCA products is also reported (Tian et al., 2006; Cheng et al., 2009). Unfortunately, the major weakness of these methods is that any nonspecific polymerization products and nucleic acid present in the sample will contribute to the fluorescence. Each method mentioned above has its own advantages and disadvantages. However, little work is focused on the features of high-throughput ability, portable device, and cost-efficient detection.

In recent years, interests are continuously increasing in the utilization of magnetic beads in bioaffinity assays, because it serves not only for a rapid and efficient separation but also as a high-throughput platform for detection. The magnetic bead has been used as a versatile tool in the separation and enrichment of nucleic acids, proteins, and other biomacromolecules (Olsvik et al., 1994; Paleček and Fojta, 2007; Yang et al., 2008; Liu et al., 2008). Meanwhile, electrochemiluminescence (ECL) detection has attracted considerable attention due to its high sensitivity, simple instrumentation, and low cost (Richter, 2004; Marquette and Blum, 2008). Very recently, the considerable advances have been made in DNA biosensors using $Ru(bpy)_3^{2+}$ with tripropylamine (TPA) ECL detection protocol (Miao, 2008; Zhu et al., 2008; Zhou et al., 2009).

In this paper, we introduce RCA assay combining ECL technology and magnetic beads for point mutation detection. The assay takes advantage of the high amplification efficiency of RCA and the intrinsically high sensitivity of ECL. Streptavidin-coated magnetic beads were used as both the separation tool and the immobilization matrix. ECL detection of magnetic beads enriched RCA products was executed *in situ* at the surface of a platinum electrode. A detection limit of 2 amol was obtained, and the positive mutation detection was achieved with a wild-type to mutant ratio of 10 000:1. The proposed RCA and magnetic bead based ECL detection strategy proved to be an efficient method for detection of point mutation.

2. Experimental

2.1. Materials

All oligonucleotides used in this work are synthesized and purified by HPLC at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (SSBE), China. Their sequences are listed in Table 1. The padlock probes are chemically 5′-phosphorylated, the capture probes are labeled with biotin for biotin–streptavidin linkage, and the detection probes are tagged with the $Ru(bpy)_3^{2+}$.

Table 1
Sequences of targets and probes in the present study.

DNA	Sequence (5′–3′)
Padlock probes	Phosphate-TGTTTGTGCCTGTCCATGTTTAACTGAATAGACTAAGACA-CTCCTTCCAGCAACTAATCAAACAG CTTTGAGGTGCA
Target-TP53-M	GTCTCTCCCAGGACAGGCACAAACATGCACCTCAA AGCTGTT CCG TCCCA
Target-TP53-W	GTCTCTCCCAGGACAGGCACAAACACGCACCTCAA AGCTGTTCCGTCCCA
Capture probes	Bio-AAAAAGATTAGTTGCTGGAA
Detection probes	TBR-TAACTGAATAGACTAAGAC

The location of the allele-specific recognition site in padlock probe and the point mutation site in target is underlined.

Streptavidin-coated magnetic beads (2.8 μm in diameter) are brought from Dynal Biotech (Lake Success, NY, USA). TPA and the chemicals to synthesize the $Ru(bpy)_3^{2+}$ N-hydroxysuccinimide ester (TBR-NHS ester) are purchased from Sigma (Louis, MO, USA). The *E. coli* DNA ligase, the exonuclease I, and 20 bp and 100 bp DNA ladder markers are from Takara Bio (Shiga, Japan). Phi29 DNA polymerase and the mixture of deoxyribonucleotides (dNTPs) are purchased from SSBE. The GoldViewtm dye is offered from SBS Genetech Co. Ltd. (Beijing, China). Other chemicals employed are of analytical reagent grade and are used as received. The high-purity deionized water (resistance >18 MΩ cm) is used in all instances.

2.2. Equipment

A custom-built ECL detection system has been described in detail in our previous reports (Tang et al., 2007; Zhu et al., 2009). The instrument consists of an electrochemical reaction cell, a potentiostat (Sanming Fujian HDV-7C), an ultra high sensitivity single photon counting module (Channel Photomultiplier, PerkinElmer MP-962), a multi-function acquisition card (Advantech PCL-836), a computer and labview software. The electrochemical reaction cell is composed of a working electrode (platinum), a counter electrode (platinum), and a reference electrode (Ag/AgCl). A magnet is located in the lower layer of work electrode to apply a magnetic field at the surface of the electrode, which allows effective enrich of the magnetic beads.

2.3. Ligation and digested reaction

For ligating of the padlock probes, target sequences were hybridized with the padlock probes in 20 μL of ligation system. The system containing 30 mM Tris–HCl (pH 8), 10 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 1.2 mM EDTA and 0.1 mM NAD, was incubated at 45 °C for 30 min. Then, 5 U of E. *coli* DNA ligase and 2 μL of 10× BSA were added to the system and incubated at 37 °C for 45 min.

In order to increase the specificity of the RCA, exonuclease I was used to digest unligated padlock probes and the linear target strands (Erik et al., 2007). Exonuclease I was added to the ligation reaction mixture and incubated at 37 °C for 40 min (Yan et al., 2004). The digestion reaction was terminated by heating at 95 °C for 5 min. The control (using the wild-type p53 sequences) was processed in parallel with the mutation target.

2.4. RCA reaction

The digested mixture was dissolved in 40 μL Phi29 reaction buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT, pH 7.5) that contained 2.5 μM biotinylated primers. The mixture was denatured at 95 °C for 5 min and cooled down to 37 °C. Then, 20 U Phi29 DNA polymerase and 500 μM dNTPs were added to the mixture, and incubated at 37 °C for 100 min. The reaction was terminated by heating the mixture at 65 °C for 10 min (Phillip et al., 2002).

2.5. ECL detection

For analyzing the samples, the 50 μL total reaction volume containing 15 μL RCA products, 10 μL detection probes (5 μM), and 25 μL hybridization buffer (20 mM TE, 600 mM NaCl, pH 7.4) was heated to 95 °C for 5 min and then incubated for 60 min at 40 °C. After the hybridization, the products were directly added to 100 μL binding buffer (10 mM TE, 500 mM NaCl, pH 7.4) containing 8 μL magnetic beads (10 μg/μL) in an eppendorf thermomixer, and then the mixture was incubated at 30 °C for 60 min. The reaction mixture was separated by using magnetic racks (Dynal, mpc-s) and washed twice with bind buffer to remove the unbound ECL probes. The

remained target analytes were resuspended in 100 μL ECL assay buffer (200 mM phosphate, 50 μM NaCl, 7 mM NaN_3, 0.8 μM Tirton X-100, 0.4 mM Tween 20, 100 mM TPA, pH 8.0). Then, the mixture was intermixed and transferred into the reaction cell, where the magnetic beads–polymerization products–TBR complexes were captured and temporarily immobilized on the working electrode by a magnet under it. A voltage of 1.25 V was applied across the electrode and the photon signal was measured (Zhou et al., 2008).

The threshold values to define a positive sample for detecting point mutation is calculated based on formula (1), where the $V_{negative}$ and $V_{stdev(neg)}$ represent the averages and standard deviations of the ECL reading from the negative control samples (using wild-type p53 gene sequence as target). A sample with an ECL value higher than the threshold values ($V_{threshold}$) is considered to be positive for mutation occurrence.

$$V_{threshold} = V_{negative} + 3V_{stdev(neg)} \quad (1)$$

3. Results and discussion

3.1. The principle of RCA–ECL for detecting point mutation

The basic principle of the proposed method is schematically showed in Scheme 1 Scheme 1. Making use of the powerfully amplified potential of RCA integrating with paramagnetic beads, we propose here a new strategy for electrochemiluminescence detection of point mutation as shown in Scheme 1A. In this method, padlock probe is a uniquely designed oligonucleotide probe which contains three distinct regions: two target complementary sequences located at the 5′ and 3′ termini and a special region hybridized with biotinylated primer. Only when the two termini of the padlock probe are perfectly hybridized with the complementary mutation target sequences, where the G base of codon 273 of TP53 oncogene is mutated into A, the DNA ligase can join the two termini to form the circular padlock probe. By contraries, the ligase cannot recognize the wild-type target (a single-base mismatch in the target DNA), resulting in the circularisation failing to proceed (see Scheme 1B). So the ligation-based RCA reaction can be employed to discriminate point mutation. Since the excess of the unligated padlock probes and TBR-tagged probes can competitively hybridize with the RCA product to lower the detection efficiency, the remaining unreacted DNA sequences are digested by exonuclease I. Following the digestion, the biotinylated primers which hybridized with a special region of resulting circular padlock probes are extended isothermally at its 3′ end by a Phi29 DNA polymerase. The long single-stranded DNA sequence is generated with tandem repeats (Nilsson et al., 2006; Laili et al., 2008; Baner et al., 1998). Subsequently, the products are hybridized with the TBR-tagged probes that are complementary with a region of repeating unit. And then the streptavidin-coated magnetic beads are added to the mixture. As a result, the products can be linked to the surface of streptavidin-coated magnetic beads through biotin–streptavidin linkage. The excess of signal probes can be easily washed off with the assist of magnetic separator. After that, the resulting magnetic beads–polymerization products–TBR complexes are resuspended in ECL assay buffer and detected in the custom-built ECL detection system.

3.2. Optimization of RCA experiment condition

The success of the current assay is primarily dependent on ligation reaction. The hybridization temperature between the two termini of padlock probe and target is a key factor for ensuring the efficient ligation reaction. So the effect of hybridization temperature was examined, as shown in Fig. 1A. In the mixture of ligation reaction, the concentration of padlock probe was set at 100 nM for

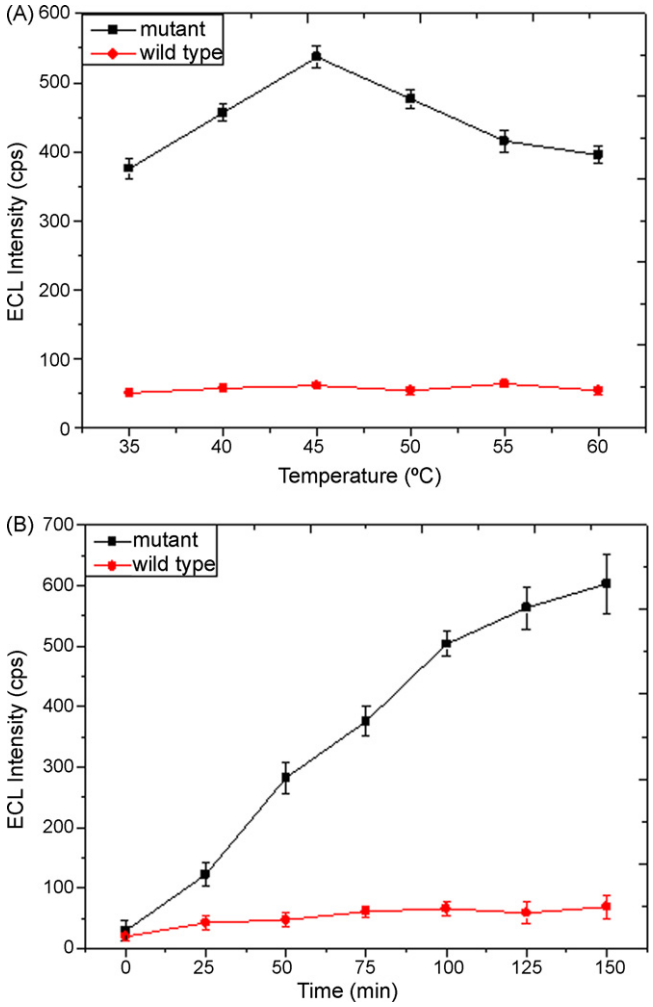
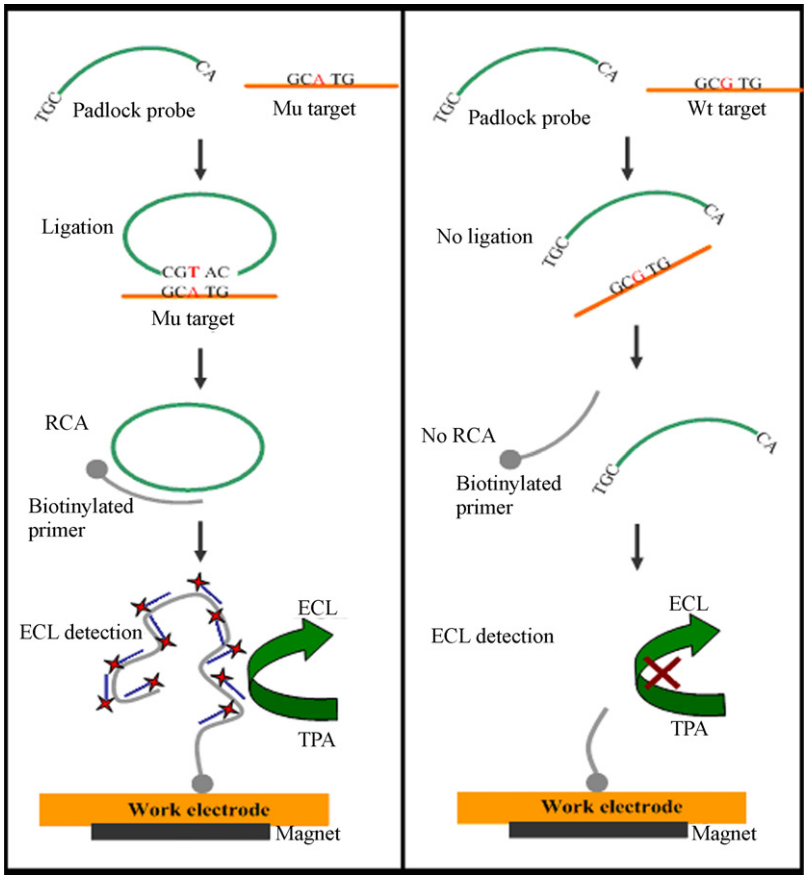


Fig. 1. Optimization of RCA experiment conditions: (A) Hybridization temperature-dependent ECL intensity changes. (B) ECL intensity–amplification time profile of the mutant and wild-type target. The ligation reaction, RCA reaction, and ECL detection are carried out as described in Section 2. Error bars show standard deviation.

all experiments in order to achieve full hybridization equilibrium to the target strands whose the concentration was varied from fM to pM. The 100 pM target sequence was added in 20 μL of reaction buffer. Then the hybridization temperature was varied from 35 to 60 °C with an interval of 5 °C. The results revealed that the maximum ECL value of mutation target was obtained at about 45 °C, while the ECL value of wild-type target was always low and changed scarcely. So 45 °C was chosen as the hybridization temperature for subsequent experiments.

In order to obtain the high assay sensitivity, the amplification duration and ECL intensity were evaluated carefully. Fig. 1B depicts the effect of RCA reaction time on the ECL readout. The amplification duration was varied from 0 to 150 min with an interval of 25 min. It was found that the ECL readout increased rapidly with the RCA reaction time up to 100 min. However, the signal exhibited no further significant increase when the reaction duration went beyond 100 min. It may be attributed to the fact that when the reaction duration increased to more than 100 min, the RCA products were entangled with each other, which can hinder the hybridization with the signal probes to some extent. In parallel assays with the wild-type target, weak ECL signal was always showed regardless of the amplification time. It indicated that the high specificity was achieved in current assay. Therefore, 100 min was selected as the optimum amplification time in the following experiments. In



Scheme 1. The principle of magnetic beads based RCA–ECL assay for highly sensitive point mutation detection. The stringent strand matching requirement of ligation discriminates single-base mismatch, and the long tandem repeated single-stranded DNA sequence is generated by RCA.

addition, the influence of pH on the performance of the ECL was investigated over the pH range of 7.5–9.5 in the hybridization system. The appropriate pH could make the DNA strands take the same charge, which could prevent the long strands entangling to a certain extent. It was favorable to enhance the efficiency of RCA products hybridizing with signal probes. An ideal pH of 8.0 was chosen for the assay experiments, which was also a general pH value in ECL detection (the data not shown).

3.3. Effect of quantity of TBR probe and magnetic beads

As an electro-active redox complex, the TBR-tagged probes would exhibit the intrinsically high sensitivity of ECL only when the probes are absorbed on the surface of electrode through specific binding to long RCA products and then react with TPA. So the influence of the quantity of TBR-tagged probes was investigated on the detection performances. As shown in Fig. 2A, the ECL value upon analysis of mutated target increased rapidly with the concentration of ECL signal probe up to 10 μM, but the value of wild-type target was always change slightly. When the probe concentration exceeded 10 μM, no further significant increase of ECL value was observed upon analysis of mutant. It was due to the fact that all the binding sites on the conglomerated RCA products were exhausted when excessive signal probes were present. The results showed that the 10 μM TBR-tagged probe was set in 50 μL the hybridization buffer as an optimized system.

In proposed method, the quantity of magnetic bead is very important to the ECL detection. The appropriate amount of magnetic beads was added in binding buffer, which was mainly used for capturing the entire biotin–polymerization products–TBR complexes. However, if excessive beads are used, the electrode would be over populated by the adsorbed beads, hence reducing the reaction of TPA and TBR on the surface of the electrode. Fig. 2B shows the effect of the quantity of beads on the ECL intensity. It was observed that the ECL response was maximized at 80 μg. On the contrary, the ECL signal obtained from wild-type was always low. Therefore, 80 μg beads were added to each detection system throughout the experiments.

3.4. The specificity of current assay

In order to evaluate the feasibility of the method, we analyzed single-base mutation in synthetic oligonucleotide targets. The wild-type target and mutant target are derived from human TP53 gene sequence. Fig. 3A displays the results obtained from target-free control (blank), 0.5 μM wild-type target, and 0.5 μM mutation target. Padlock probes concentration was set at 0.5 μM in this assay. The reaction and detection were carried out under the optimized conditions. The average ECL value obtained from analysis of the mutation target was 3421.97 ± 265.38 counts per second (cps), which was significantly higher than the value of wild-type target (218.56 ± 16.98 cps). This data demonstrated that the proposed method could be used to discriminate between the two alleles markedly.

The specificity of the ligation reaction was also validated by electrophoresis. The resulting product of the ligation reaction was firstly analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) stained with a standard silver-staining method. In Fig. 3B, the M lane represents 20 bp DNA ladder, the lanes 1 and 2 represent the ligated product from the mutation and wild-type target, respectively. It is showed that the linear padlock probe (77 bp) was ligated into a circular form only in the presence of mutated target.

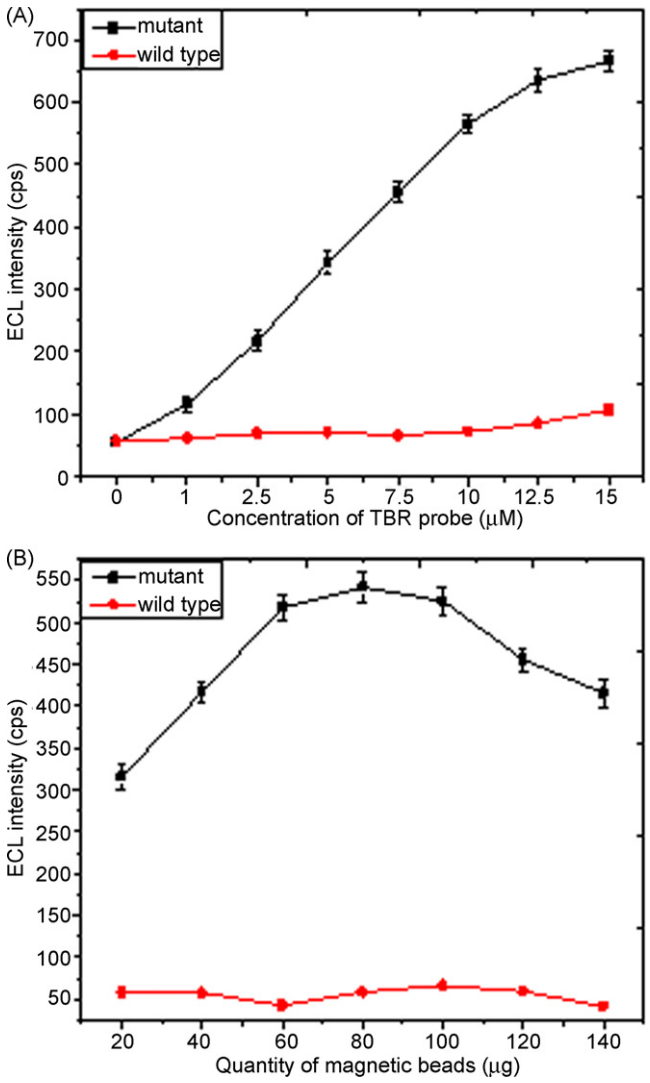


Fig. 2. (A) The effect of the quantity of TBR probe on ECL intensity. (B) The effect of the quantity of magnetic beads on the ECL intensity. All reactions are carried out as described in Section 2. Error bars show standard deviation.

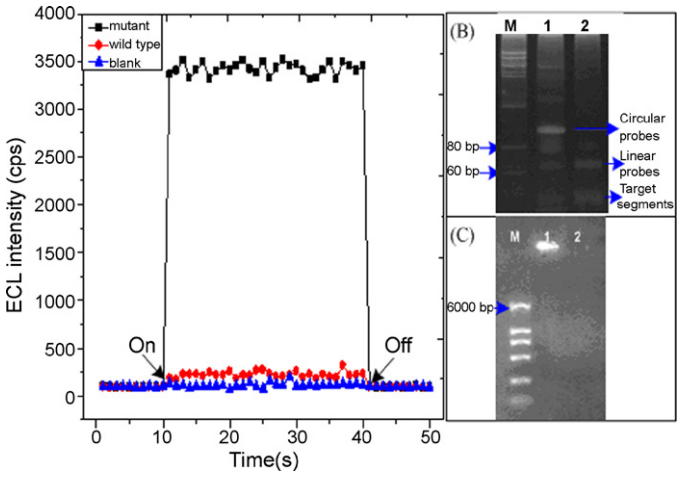


Fig. 3. Specificity evaluation of RCA–ECL method. (A) ECL intensities corresponding to target-free control, 0.5 μM wild-type and 0.5 μM mutant target. On: potentiostat on. Off: potentiostat off. (B) Electrophoretic identification of the enzymatic ligation reaction. The ligation products are separated by 10% ploayacrylamide gel electrophoresis containing 8 M urea, and DNA is screened with a standard silver-staining method. The 20-bp ladder is indicated in lane M. Lanes 1 and 2 represent the ligation products from mutation and wild-type, respectively. (C) RCA products are electrophoresed in a 1% agarose gel stained with GoldView™ dye. The DNA ladder is indicated in lane M. Lanes 1 and 2 represent the RCA products derived from the mutated target and wild-type target.

In contrast, no ligated products were obtained in the presence of wild-type target, indicating that the ligation reaction was highly specific for single-base discrimination. Subsequently, the resulting products of RCA were electrophoresed in a 1% agarose gel stained with GoldView™ dye and observed under the image analysis software (Quantity One™, Bio-RAD, CA, USA). In Fig. 3C, the M lane represents DNA ladder, the lanes 1 and 2 represent the amplified product from the mutation and wild-type targets, respectively. There is an obvious strap appeared in the mutant, but the strap is not found in the wild-type. It demonstrated that the long single-strand DNA sequence could be produced with a high specificity. The results of gel electrophoresis were consistent with the results of ECL detection.

3.5. The sensitivity of point mutation detection

To evaluate the applicability of this method, the sensitivity is regarded as one of the most crucial parameters. We mixed the different concentrations of target with invariable 100 nM padlock probes in 20 μL of reaction buffer, and then the ligated circular templates were amplified. As shown in Fig. 4A, the ECL intensity decreased sharply when the target concentration decreased from 1 nM to 100 pM. As the concentration decreased continuously, the decrease of the ECL intensity slowed down gradually. On the contrary, the low ECL value from the analysis of the wild-type target had altered scarcely. The results showed a high sensitivity and specificity obtained from the proposed method, even when the target concentration decreased to 0.1 pM (2 amol mutated sequences in 20 μL system). At this concentration, the ECL value of mutant and wild-type was 178.78 ± 13.46 cps and 69.52 ± 6.23 cps, respectively. According to the formula (1), the threshold value was set as 88.21 cps, indicating the mutation target could be discriminated easily. To investigate the precision of the proposed assay, relative standard deviation (RSD) was determined by measuring ECL signal at 0.1 pM and 1 nM mutation target with six replicates, and RSD values for both low and high target concentration were less than 10%, suggesting an acceptable reproducibility.

It is believed that the sensitivity of the current assay is comparable with or even exceeded the sensitivity of other reported RCA based methods, such as the reported branched RCA detection of RNA with a sensitivity of 6 amol (Cheng et al., 2009), 2 fmol of CE-RCA assay (Li et al., 2008b), 0.18 fmol of chemiluminescent combined with RCA (Li et al., 2008a), fluorescence detection based on RCA with a sensitivity of 6.4 fmol (Guo et al., 2009), and microsphere-based RCA with a sensitivity of 10 amol. In the proposed assay, the detection limit of 2 amol is achieved. The high sensitivity can be attributed to the following factors. For instance, the Phi29 DNA polymerase has shown a strong and reliable ability to displace newly synthesized DNA strands under isothermal conditions, so the long single-stranded DNA sequences are produced with tandem repeats. And a large number of signal probes can be bound to the single-stranded sequences for ECL detection. In addition, the magnetic beads–polymerization products–TBR complexes can readily be collected on the electrode surface by using a magnet under the electrode. Then the ECL detection of magnetic beads enriched biotinylatical RCA products is executed *in situ* at the surface of a platinum electrode, leading to a construction of a highly condensed $Ru(bpy)_3^{2+}$ domain. So the high sensitivity can be achieved. Otherwise, the complexes are collected on the electrode surface only by using a magnet without any modification on surface of electrode. Therefore, the electrode can be reused by simply washing out the beads from the sur-

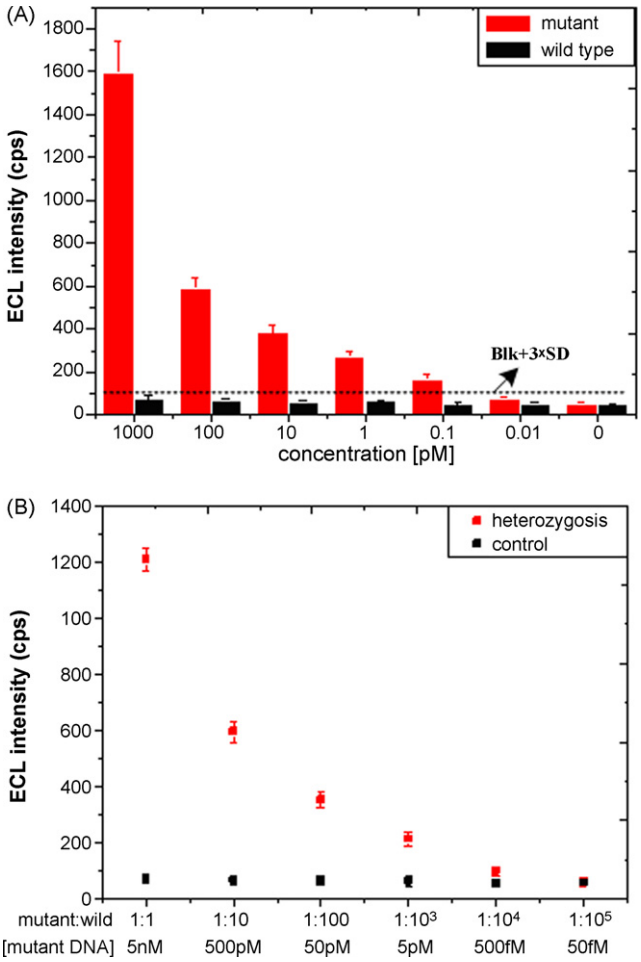


Fig. 4. (A) The sensitivity assessment of RCA–ECL method. The dashed line represents the threshold value calculated based on formula (1). (B) Analysis of heterozygous samples. ECL values and error bars represent the reading from independent assays and standard deviation. The ligation reaction, RCA reaction, and ECL detection are carried out as described in Section 2. Error bars show standard deviation.

face, resulting in a rapid detection process and a reduced detection cost.

The specificity of the proposed method was further evaluated for detecting mutations present at a low percentage in a mixed heterozygous sample. Evaluation of this performance is important, since in tumor samples mutated alleles frequently represent only a fraction of the allelic population (nontumoral gene in the analyzed biopsies, heterogeneity of the tumoral cells, heterozygous mutations). The better the performance, the higher the ability to detect a small proportion of mutated gene within the surrounding wild-type gene. To do this, we mixed samples of the mutation sequences and wild-type sequences together with different mole ratios (1:1, 1:10, 1:100, 1:1000, 1:10 000, and 1:100 000). The wild-type target was always at a concentration of 5 nM, and the final concentration of mutation target was varied from 5 nM to 50 fM. The results were displayed in Fig. 4B, the ECL intensity decreased rapidly when the mole ratio decreased from 1:1 to 1:100. With the continued decrease of the mole ratio, the decrease of the ECL intensity slowed down obviously. When the mole ratio decreased to 1:10 000, the signal of mutation target was 108.78 ± 12.51 cps and the signal from absolutely wild-type target was 74.60 ± 7.99 cps. According to the threshold value (98.6 cps), detection limit down to 0.5 pM was achieved in heterozygous samples (a mutant to wild-type ratio of 1:10 000), which could be attributed to the high fidelity of DNA ligase in distinguishing mismatched bases at the ligation site. It indicated that the proposed method had an extremely high selectivity with rather low limit of detection (LOD), which made possible to have a potential clinical use in the future.

4. Conclusions

In the present work, we have developed a new method to detect point mutation with p53 as a model. The high sensitivity and specificity of the new method can be mainly attributed to the specific ligase assay, robust amplification potential of RCA, and intrinsically high sensitivity of ECL. As low as 0.1 pM mutated target has been detected by this assay, and positive mutation detection is achieved with a mutant to wild-type ratio of 1:10 000. The developed method holds some advantages over other techniques, such as simplicity and high specificity. Furthermore, since RCA can avoid complicated thermal cycling steps (an isothermal reaction at 37 °C), heating in a period of time (90 min) using a simple incubator such as a water bath or a block heater is sufficient to amplify DNA to detectable levels. Combining with the magnetic beads based ECL technique, the proposed assay can be easily extended to a high-throughput and automatic screening format. This capability is indeed a desired feature for future clinical application. Eventually, we anticipate that this technique will be broadly applicable for the detection of a variety of biorelated markers.

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