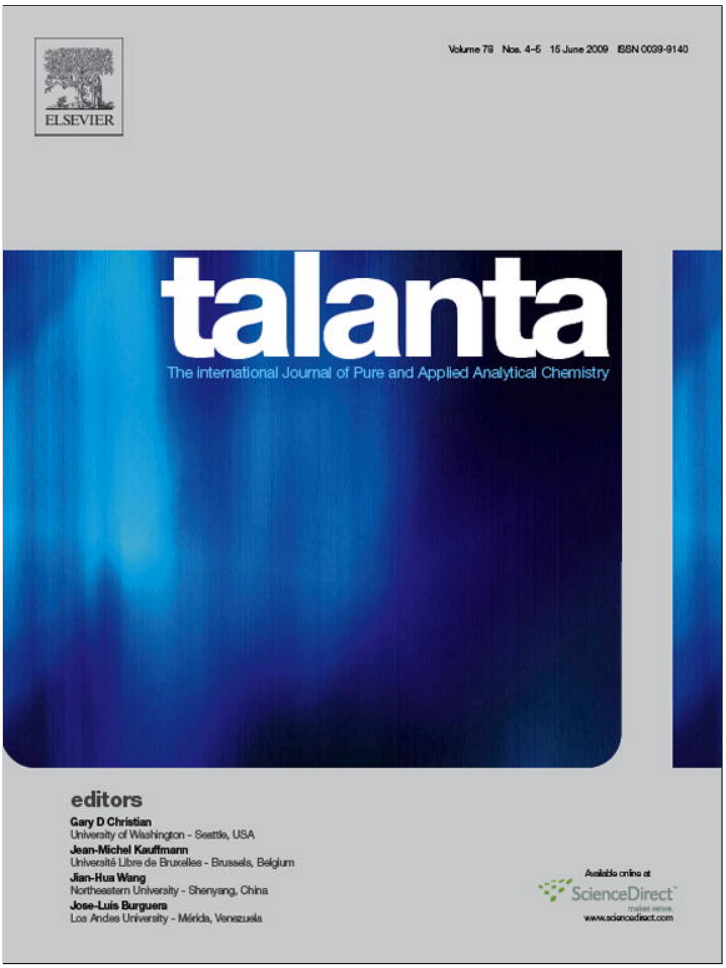

Talanta 78 (2009) 1253–1258

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Rapid and sensitive detection of point mutation by DNA ligase-based electrochemiluminescence assay

Huijuan Zhou, Da Xing*, Debin Zhu, Xiaoming Zhou

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, South China Normal University, Guangzhou 510631, China

ARTICLE INFO

Article history:
Received 8 November 2008
Received in revised form 16 January 2009
Accepted 18 January 2009
Available online 24 January 2009

Keywords:
Point mutation
Electrochemiluminescence
Oligonucleotide ligation assay

ABSTRACT

The identification of single-base mutations in particular genes plays an increasingly important role in medical diagnosis and prognosis of genetic-based diseases. Here we report a new method for the analysis of point mutations in genomic DNA through the integration of allele-specific oligonucleotide ligation assay (OLA) with magnetic beads-based electrochemiluminescence (ECL) detection scheme. In this assay, the tris(bipyridine) ruthenium (TBR) labeled probe and the biotinylated probe are designed to perfectly complementary to the mutant target; thus a ligation can be generated between those two probes by Taq DNA Ligase in the presence of mutant target. If there is an allele mismatch, the ligation does not take place. The ligation products are then captured onto streptavidin-coated paramagnetic beads, and detected by measuring the ECL signal of the TBR label. Results showed that the new method held a low detection limit down to 10 fmol and was successfully applied in the identification of point mutations from ASTC-α-1 cell line, PANC-1 cell line and blood cell in codon 273 of *TP53* oncogene. In summary, this method provides a sensitive, cost-effective and easy operation approach for point mutation detection.

1. Introduction

The analysis of genomic mutations in disease-related gene fragments is playing an increasingly important role in fields of genetic-based diseases diagnosis and drug reaction prediction. Since large numbers of mutations must be evaluated in order to obtain an accurate diagnosis/prognosis of that disease, a sensitive, rapid and cost-effective DNA identification method is in need. A variety of technologies based on allele discrimination strategies have been applied in the identification of point mutations, such as primer extension [1–4], allele-specific hybridization [5–8], enzymatic cleavage [9,10], and oligouncleotide ligation [11–13]. Among these allele-discrimination strategies, DNA enzyme-based assay is popular because it is highly specific, cost effective, and easy to operate and fast to implement. In these enzyme-based assays, DNA ligase is an enzyme which is used frequently in the discrimination of point mutations [14–17], insertions and deletions [18]. Several conventional detection methods, such as denaturing gradient gel-electrophoresis [19,20], mass spectrometry [21,22] and fluorescence signal-based detection [23,24] have been used to the ligase based strategy. These technologies provide accurate or highly sensitive approaches for point mutation detection. However, each of them still has its disadvantages. For example, some of them are time consuming, complicated, or requiring special instruments and the use of expensive fluorescent substances. Recently, methods based on chemiluminescence [25,26] and electrochemistry [27,28] have also been used to facilitate single-base mutation identification for the advantages of rapidness and cost efficiency.

In recent years, electrochemiluminescence (ECL) has attracted considerable attention due to its feature of high-sensitivity, low-cost, simple instrumentation, and time efficiency. The most common ECL luminophore is tris(2,2-bipyridine)ruthenium(II) (TBR), and tripropylamine (TPA) is the most efficient known co-reactant. In this ECL reaction, TBR and TPA are first oxidized at the surface of an anode forming the strong oxidant $Ru(bpy)_3^{3+}$ and the cation radical $TPA^{\bullet+}$, respectively. The resulted $TPA^{\bullet+}$ immediately loses a proton and becomes a powerful reducer, $TPA^{\bullet}$. Then $Ru(bpy)_3^{3+}$, a strong oxidant, and $TPA^{\bullet}$, a strong reductant, react to form the excited state of the ruthenium complex, $Ru(bpy)_3^{2+*}$, as well as other inactive products. Relaxation of the excited-state $Ru(bpy)_3^{2+*}$ to the ground state results in a light emission, at 620 nm [29,30]. It should be noticed that $Ru(bpy)_3^{2+}$ is not consumed during the reaction, and may be oxidized and excited repeatedly when there is excessive TPA in the buffer. Since Kenten et al. [31] firstly used ECL in DNA probe assays, the technology has been widely applied in the areas of DNA analysis [32], immunoassay, food and water testing and biowarfare agent detection [33].

In this paper, we describe a specific and sensitive method for point mutations assay which is accomplished by incorporating OLA into magnetic beads-based ECL detection scheme. Taq DNA ligase is applied for single-base mutation discrimination through catalyzing the joint of the common probe and the discriminating probe

* Corresponding author. Tel.: +86 20 85210089; fax: +86 20 85216052.
E-mail address: xingda@scnu.edu.cn (D. Xing).

0039-9140/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2009.01.032

Table 1
Oligonucleotides used in this assay.

Oligonucleotide	Sequence (5′–3′)
TP53 exon 8 forward	5′-CTGATTTCCTTACTGCCTCTTG-3′
TP53 exon 8 reverse	5′-TACCTCGCTTAGTGCTCCCT-3′
TBR-labeled probe	5′-TBR-$(CH_2)_6$-TGGGACGGAACAGCTTTGAGGTGCA-3′
Biotin-labeled probe	5′-Phosphate-TGTTTGTGCCTGTCCTGGGAGAGAC-Biotin-3′
Target 1 (Ml)	5′-CCAGGACAGGCACAAACATGC ACCTCAAAGCTGTT-3′
Target 2 (Wl)	5′-CCAGGACAGGCACAAACACGCACCTCAAAGCTGTT-3′

The location of the allele-specific recognition site in TBR-labeled probe and the polymorphism sites in target is underlined.

to form a single oligonucleotide in the presence of perfect match target DNA. Ligation products are then captured onto streptavidin-coated paramagnetic beads through biotin-streptavidin interaction, and then the luminescence signal will be detected by ECL system. According to the signal we can determine whether the sample is mutant type or wild type. This method is proven to be effective in analyzing the point mutation in codon 273 of *TP53* oncogene target from PANC-1 human pancreatic cancer cell line, ASTC-α-1 lung cancer cell line, and blood cells.

2. Experimental

2.1. Oligonucleotides and reagents

The primers (Table 1) for PCR amplification were designed using Primer Premier 5 software and the two probes (Table 1) used in DNA ligase reaction were designed with the help of DNA probe design software (Zucker folding program, www.bioinfo.rpi.edu/applications/mfold/dna/form1.cgi). The mutant target (M1, Target 1), wild-type target (W1, Target 2) and the primers, the 5′-amino modified probe (TBR labeled probe) and the other probe which is labeled with biotin at 3′ and phosphate at 5′ probe (Table 1) were synthesized by Shanghai Sangon Biological Engineering & Technology Services Co. Ltd. (SSBE), China. All probes were HPLC purified. TPA, and the chemicals to synthesize the $Ru(bpy)_3^{2+}$ N-hydroxysuccinimide ester (TBR-NHS ester) were products of Sigma (Louis, MO, USA). TBR-NHS ester was synthesized by our laboratory according to Terpetschnig's paper [34]. Streptavidin microbeads (2.8 μm diameter) were purchased from Dynal Biotech (Lake Success, NY, USA). Taq DNA ligase was purchased from New England BioLabs, Inc. (Beverly, MA). Taq DNA polymerase, dNTP, 2000 bp DNA Marker and the UNIQ-10 column genome DNA extraction kit were all purchased from SSBE. All other reagents were of analytical grade.

2.2. Extraction of DNA from cell lines

Genomic DNA was extracted from PANC-1 human pancreatic cancer cell line, ASTC-α-1 lung cancer cell line and blood cells. The PANC-1, ASTC-α-1 cell lines were cultured as described in our recent papers [35,36].

The blood samples were obtained from healthy donors. 1 mL of ACD decoagulant (0.48% citric acid, 1.32% sodium citrate, 1.47% glucose) was added to each 6 mL blood. 500 μL of blood was diluted by adding 1 mL sterile distilled water, and then was centrifuged at 5000 rpm for 2 min at 25 °C. The sediment was resuspended with 200 μL Tris–EDTA (TE) buffer (pH 8.0) for DNA extraction.

DNA was extracted according to the protocol of the UNIQ-10 column genome DNA extraction kit.

2.3. PCR amplification of genomic DNA

The extracted genomic DNA was amplified using the primers (Table 1) specific for exon 8 of *TP53* gene in a total volume of 50 μL. Each reaction contained 5 μL of 10 × buffer, 1 μL of 10 μM each amplification primers, 1 μL of 10 mM dNTP mixture, 1 U of Taq DNA polymerase, and 1 μL of extracted DNA. After a 5 min denaturation step, the application was achieved by thermal cycling for 35 cycles at 95 °C for 1 min, 60 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min.

2.4. Oligonucleotide ligation assay

For oligonucleotide ligation assay, a 20 μL reaction mixture containing template (synthetic oligonucleotide targets or PCR products) in different concentrations, 1 μM biotin-labeled probes, 1 μM TBR-labeled probes and Taq ligation buffer [20 mM Tris–HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD^+, 0.1% Triton X-100] was prepared. The mixture was denatured for 5 min at 95 °C, hybridized for 60 min at 55 °C, followed by adding of 2 U of Taq DNA ligase, and then the ligase reaction took place at 45 °C for 30 min.

2.5. ECL detection

A custom-made ECL detection system was described in detail in our previous research [29]. For the sample analysis, 10 μL OLA products and 10 μL streptavidin coated beads were added to 80 μL bind buffer (10 mM TE, 500 mM NaCl, pH 7.4), and then incubated in an Effendorf thermomixer for 30 min at 30 °C. The reaction products were separated by using magnetic racks (Dynal, mpc-s), and washed first with 50 mM NaOH and then with bind buffer to remove the unbound probes labeled with TBR. Then the magnetic-ligation products were resuspended in 100 μL ECL assay buffer (0.2 M NaH_2PO_4, 50 μM NaCl, 7 mM NaN_3, 0.8 μM Tirton X-100, 0.4 mM Tween 20, 0.1 M TPA, pH 8.0) and was sequentially used for ECL detection. 50 mM NaOH solution was used to denature the duplex and dissociate the DNA template, retaining the DNA fragments contained biotin on the streptavidin coated magnetic beads. 50 mM NaOH can denature DNA duplexes with 1 min whereas biotin-streptavidin bind is essentially undisturbed under this condition [37,38]. The applied potential of the ECL reaction was fixed at 1.25 V and the ECL system was controlled by Labview software.

3. Results and discussion

3.1. Assay principle

The detection principle is illustrated in Fig. 1. In the design, common probes modified with biotin at 3′ end and discriminating probes modified with TBR at 5′ end. Both probes hybridize with the target DNA at adjoining positions. Since the probes we designed are only perfectly complementary to the mutant template DNA, Taq DNA ligase can specially catalyze the formation of a phosphodiester bond between the juxtaposed 5′-phosphate and 3′-hydroxyl groups of above noticed probes to form a single oligonucleotide in the presence of mutant target DNA, but no ligation occurs for probes associated with the wild-type template. After ligation reaction, the products are concentrated by streptavidin-coated magnetic beads through the highly selective biotin-streptavidin reaction. The unlinked DNA fragments are washed away, thus, only the products labeled with both biotin and TBR can be detected in the detection cell.

3.2. Optimization of assay conditions

The hybridization temperature between the probes (biotin-labeled probe and TBR-labeled probe) and target DNA in analysis of targets is an important factor. As we know, there will be a melting temperature (T_m) which means half of the hybridized DNA duplex

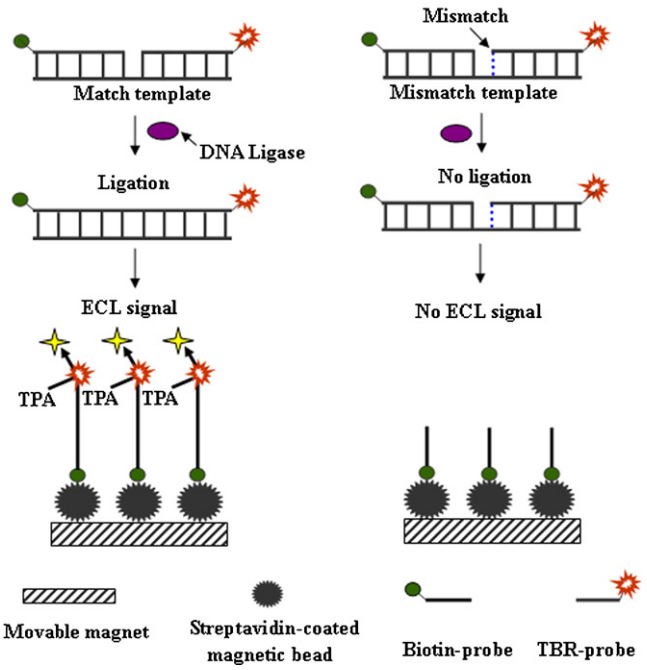

Fig. 1. Schematic for point mutation detection based on OLA and magnetic beads-based ECL detection scheme. Biotin-probe: biotin labeled probe. TBR-probe: TBR labeled probe.

melted at this temperature. The T_m between two designed probes (biotin-labeled probe TBR-labeled probe) and the target DNA is 65 and 68 °C respectively, according to DNA probe design software (Zucker folding program) and the theoretical calculation [39]. In common practice, the temperature approximately 10 °C lower than the melting temperature is chosen as the optimal Hybridization temperature. So, the hybridization temperature was fixed at 55 °C in the detection system. At the relatively higher temperature, the formed duplex DNA will melt. However, if oligonucleotides hybridize at relatively lower temperature, there will be nonspecific hybridization.

The effect of hybridization time on the ECL intensity was also investigated, to ensure the method timesaving. A mixture containing each probes and 10 nM target incubated at 55 °C for various hybridization times. As shown in Fig. 2, the ECL intensity response increased rapidly with the hybridization time up to 1 h. After 1 h, the ECL intensity could not be further improved, indicating that the hybridization equilibrium was reached at 1 h. Therefore, a

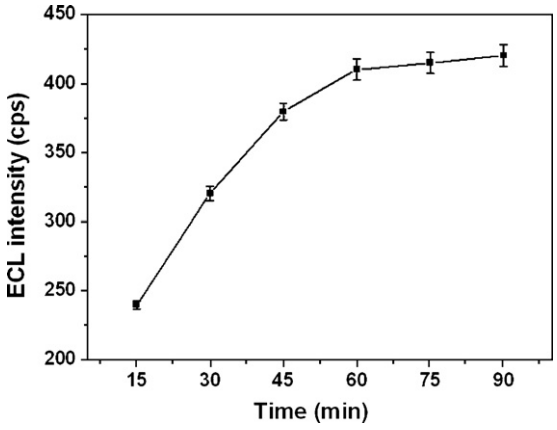

Fig. 2. The effect of hybridization time on the ECL intensity. The ECL intensity was plotted vs. hybridization time.

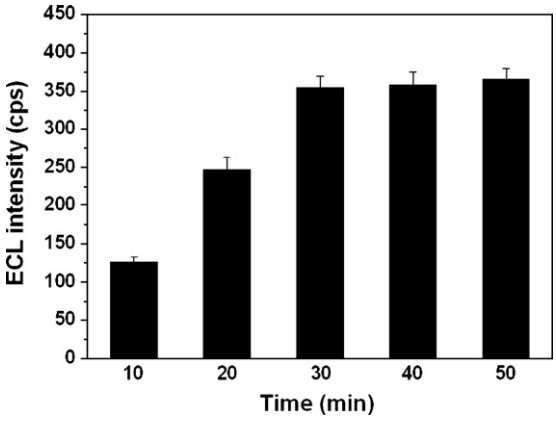

Fig. 3. The effect of ligase deposition time on the ECL intensity. The response represents the ECL intensity change induced by the deposition time changed from 10 min to 50 min for the ligation reaction. The concentrations of synthetic mutant template is 10 nM.

hybridization time of 1 h was selected as the optimum in the following experiments.

We also investigated the effect of ligase reaction time on the ECL intensity. After hybridized at 55 °C for 1 h, the Taq DNA ligase was added to the reaction solution and incubated at 45 °C for different times varying from 10 to 50 min. As shown in Fig. 3, the ECL intensity increased rapidly until 30 min. After 30 min, ECL intensity increased slowly. That phenomenon is mostly probably because Taq DNA ligase lost its activity with the increase in the reaction time. So the reaction time was fixed at 30 min throughout the experiment.

In the ECL detection, the quantity of magnetic beads is a vital factor [40]. Since the appropriate amount of beads can capture the entire special ligation products, thus improving the sensitivity. But excessive beads would be absorbed on the surface of electrode, and influence the reaction of TPA and $Ru(bpy)_3^{2+}$ on the surface of electrode. In the experiments, the ligation products were linked on to the surface of streptavidin-coupled beads by the biotin modified on the common probe at the 3′ end through the highly selective biotin-streptavidin linkage. The unlinked DNA fragments were washed away. As shown in Fig. 4, the quantity of magnetic beads from 4 to 14 μL gave the different ECL responses, and it is observed that the ECL response is maximized at a beads quantity of 10 μL. Therefore, 10 μL beads were added to each 10 μL ligation product throughout the experiment.

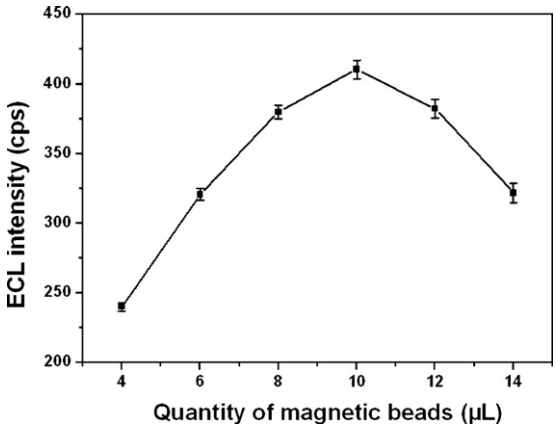

Fig. 4. The effect of the quantity of streptavidin-coupled magnetic beads on the ECL intensity. The concentration of the mutant target is 10 nM, hybridization time is 1 h and ligase deposition time is 30 min.

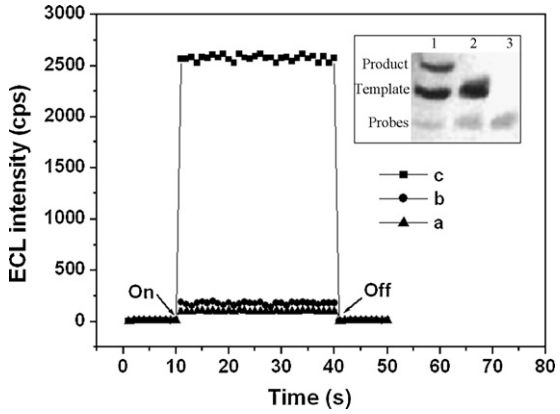

Fig. 5. Specificity evaluation of OLA-ECL method. ECL intensity corresponding to (a) template free control, (b) 1 μM wild-type template, (c) 1 μM mutant template. 1 μM ligation products were separated by 10% ploy acrylamide gel electrophoresis containing 8 M urea, and DNA was screened with a standard silver-staining method. (1) Mutant ligation products, (2) Wild-type liagtion products, (3) Template free ligation products control. On: potentiostat on. Off: potentiostat off.

3.3. Analysis of single-base variations in synthetic oligonucleotide targets

In order to evaluate the feasibility of the method, we firstly analyzed single-base mutation in synthetic oligonucleotide templates (Table 1), to avoid the effects of variability between real DNA samples on the evaluation results. Wild-type template (W1) and mutant template (M1) were derived from variants of human *TP53* gene sequence. Fig. 5 displays the results obtained from template free control (a), 1 μM wild-type template (b), 1 μM mutant template (c). It was observed that the ECL signal obtained from analysis of perfectly matched mutant target (2568 cps) was significantly higher than that of wild-type target (177 cps) with a single-base mismatch. As a result, the signal-to-noise ratio in discriminating point mutation reaches 14.5 in the proposed method, demonstrating that the proposed method based on Taq DNA ligase reaction can be used to distinguish mutant target DNA and the wild target DNA efficiently. The incorporation of OLA not only provides high specificity but also is easy to operate. The application of DNA ligase reaction for single base mismatch identification substantially improved the capacity of point mutation discrimination without tight control of the assay conditions. In contrast, the application of specific DNA sequence based on DNA hybridization assay for point mutation detection requires stringent temperature control in hybridization reaction [5–8]. In mutation detection, specificity and operational ease are of the most significance. To further validate the accuracy of the current method, the ligation products were analyzed by 10% polyacrylamide gel electrophoresis. As shown in Fig. 5, there is an obvious product band appeared in mutant ligation products, but without product band was found for the wild-type ligation products. Thereby, the results of gel electrophoresis are consistent with the results of ECL detection.

Under the optimal experimental conditions, the sensitivity of the reported system was evaluated through analysis of various quantities of the perfectly matched and mismatched targets (quantity banding from 10 pmol to 1 fmol). In order to avoid cumulated background, this assay started from low quantity to high quantity. As depicted in Fig. 6A. The proposed method offers well-defined concentration dependence. It was found that when the quantity of target was down to 10 fmol, the wild-type target and mutant target can be distinguished easily. The detection limit is 10 times higher than that shown previously [36]. Furthermore, the technique is much simpler than conventional DNA detection methods. This is attributed to the application of streptavidin-coated paramagnetic

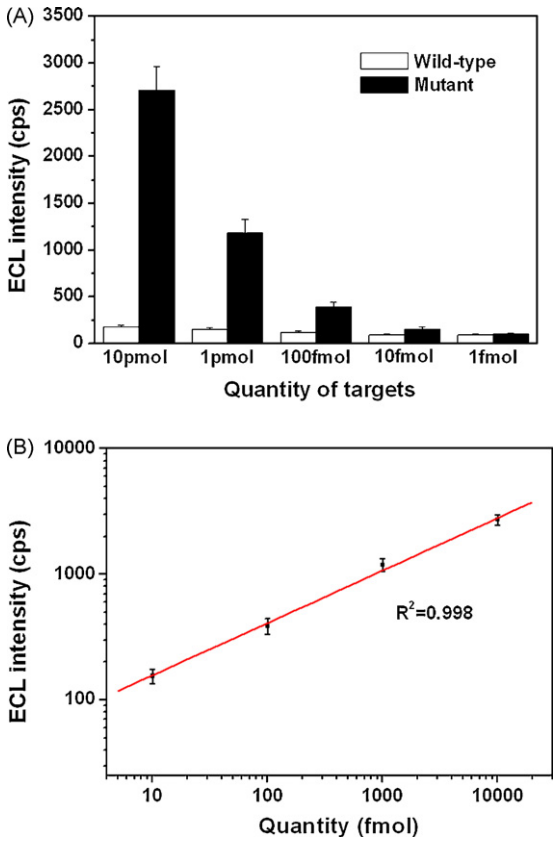

Fig. 6. Sensitivity and linearity of OLA-ECL method. (A) ECL intensity as a function of the sensitivity for detecting perfectly matched and mismatched templates of various concentrations varying from 10 pmol to 1 fmol. (B) Linearity of ECL intensity was observed in different quantities of mutant template DNA with OLA-ECL.

beads. In the reported method, streptavidin-coated paramagnetic beads were specifically chosen for selective capture of biotinylated ligation products. The binding interaction of streptavidin and biotin is quick, reliable and strong ($Kd = 10^{-15}$), and dramatically reduces the time of sample preparation. Otherwise, in ECL detection, the magnetic beads-ligation products complex can readily be collected on the electrode surface only by using a magnet without any modification on electrode surface. This ensures that the electrode can be reused by simply washing out the beads from the surface, resulting in a rapid detection process and a reduce detection cost. Moreover, the method can be easily extended to high-throughput and automatic screening format with the use of the magnetic beads. The calibration plot depicted in Fig. 6B shows that the ECL intensity exhibit an excellent linear relationship to the quantity of mutant target DNA in the range from 10 pmol to 10 fmol and the correlation coefficient is 0.998. It is noteworthy that our detection method covers a dynamic range of at least two orders of magnitude.

3.4. Analysis of TP53 point mutation

In order to further validate the OLA-ECL method, we applied the method to detect point mutations in PCR products from cell lines. *TP53* point mutation at codon 273 was chosen as the target. *TP53* gene is one of the most studied genes in cancer research and the role of *TP53* as an important early diagnostic marker of tumours has been suggested [41]. As shown in Fig. 7A, the ECL signals obtained from PCR products of PANC-1 human pancreatic cell lines, ASTC-α-1 lung cancer cell lines and blood cells are 262.2 ± 13.4 cps, 24.3 ± 2.9 cps, 26.4 ± 3.6 cps, respectively. PCR

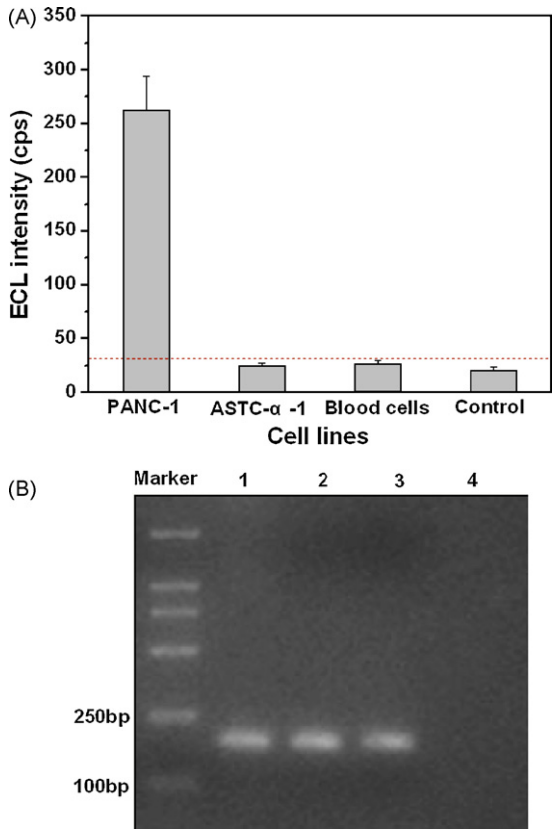

Fig. 7. Detection of real cell line samples. (A) ECL intensities obtained by analysis of single-base mutation at codon 273 of *TP53* gene in PCR products (186 bp) from PANC-1, ASTC-α-1, normal blood cells, and without template control. All ECL signal value were subtracted the blank control signal (ECL assay buffer). The red dash line represents the cut-off value for mutation positive samples. (B) Gel electrophoresis analysis the PCR products of PANC-1 cells (1), ASTC-α-1 cells (2), normal blood cells (3), and template free control (4).

reaction solution without template was as the control, the signal of which is 20.1 ± 3.5 cps. To define if a sample is mutation-positive, a cut-off value was calculated based on the average ($V_{control}$) and standard deviation ($V_{stdev(con)}$) of the ECL reading from the control sample, shown as in the following formula:

$$V_{cutoff} = V_{control} + 3V_{stdev(con)} \quad (1)$$

According to this formula, the cut-off level for mutation-positive samples was set at 31 cps. ECL signal less than 31 cps should not be indicated as mutation-positive under our conditions. According to the data, only PANC-1 is mutation-positive sample, and there is no G → A point mutation at codon 273 of *TP53* gene in ASTC-α-1 cell lines and blood cells. It was implied that the PCR template only from PANC-1 could perfect match with the probes at the mutation site then the two adjacent probes were integrated to form a single-stand oligonucleotide. That is to say, the codon 273 of *TP53* gene is mutant from CGT to CAT in PANC-1 cell lines. To validate if the difference of ECL intensity comes from the effect of concentration of real DNA samples and the PCR efficiency, 1% agarose gel electrophoresis analysis for PCR products was performed in the experiment. Fig. 7B shows the concentrations of the PCR products (186 bp) from PANC-1 cell line (line 1), ASTC-α-1 cell line (line 2) and blood cells (line 3) are almost the same. Without strap was observed in the PCR control (line 4) indicated the PCR process was fairly specific. Therefore the proposed approach could potentially serve as robust assay for mutation detection.

4. Conclusions

We reported a novel simple and sensitive approach for DNA point mutation detection. The approach bases on the high-fidelity perfect-match ligation by OLA and magnetic beads-based ECL detection scheme. Taq DNA ligase was used to offer very high fidelity in identification of template gene containing single-base mutation, which was proved to be efficient and specific. The magnetic beads-based ECL detection scheme provides the assay system with simple operation, cost-efficiency and significantly sensitivity. Thereby the proposed OLA-ECL approach could be potentially applied in the field of genetic-based diseases clinic early diagnosis and drug response prediction as well as point mutation detection in cancer studies.

Acknowledgements

This research is supported by the National Natural Science Foundation of China (30600128, 30870676, 30800261), the Natural Science Foundation of Guangdong Province (7005825), and the National High Technology Research and Development Program of China (863 Program) (2007AA10Z204).

References

[1] B.P. Sokolov, Nucleic Acids Res. 18 (1990) 3671.
[2] R.A. Gibbs, P.N. Nguyen, C.T. Caskey, Nucleic Acids Res. 17 (1989) 2437.
[3] S. Sauer, I.G. Gut, Rapid Commun. Mass Spectrom. 17 (2003) 1265.
[4] S. Bortolin, M. Black, H. Modi, I. Boszko, D. Kobler, Clin. Chem. 50 (2004) 2028.
[5] J.G. Hacia, B. Sun, N. Hunt, K. Edgemon, D. Mosbrook, C. Robbins, S.P. Fodor, D.A. Tagle, F.S. Collins, Genome Res. 8 (1998) 1245.
[6] S. Tyagi, D.P. Bratu, F.R. Kramer, Nat. Biotechnol. 16 (1998) 49.
[7] G.C. Kennedy, H. Matsuzaki, S. Dong, W.M. Liu, J. Huang, Nat. Biotechnol. 21 (2003) 1233.
[8] S. Haasl Russom, A.J. Brookes, H. Andersson, G. Stemme, Anal. Chem. 78 (2006) 2220.
[9] D. Botstein, R.L. White, M. Skolnick, R.W. Davis, Am. J. Hum. Genet. 32 (1980) 314.
[10] V. Lyamichev, A.L. Mast, J.G. Hall, J.R. Prudent, M.W. Kaiser, Nat. Biotechnol. 17 (1999) 292.
[11] U. Landegren, R. Kaiser, J. Sanders, L. Hood, Science 241 (1988) 1077.
[12] F. Barany, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 189.
[13] P. Hardenbol, F. Yu, J. Belmont, J. Mackenzie, C. Bruckner, et al., Genome Res. 15 (2005) 269.
[14] N.P. Gerry, N.E. Witowskl, J. Day, R.P. Hammer, G. Barany, F. Barany, J. Mol. Biol. 292 (1999) 251.
[15] X.X. Meng, H.M. Li, K.M. Wang, W.H. Tan, J. Li, Q.P. Guo, X.H. Yang, Q.H. Mo, X.M. Xu, Talanta 73 (2007) 23.
[16] H.X. Tang, X.H. Yang, K.M. Wang, W.H. Tan, H.M. Li, L.F. He, B. Liu, Talanta 75 (2008) 1388.
[17] P. Zhang, X. Chu, X.M. Xu, G.L. Shen, R.Q. Yu, Biosens. Bioelectron. 23 (2008) 1435.
[18] R. Favis, J.P. Day, N.P. Gerry, C. Phelan, S. Narod, F. Barany, Nat. Biotechnol. 18 (2000) 561.
[19] S. Dieter, B. Alexei, A.N. Mark, Nucleic Acids Res. 28 (2000) e43.
[20] S.A. Brazill, W.G. Kuhr, Anal. Chem. 74 (2002) 3421.
[21] J. Tost, I.G. Gut, Mass Spectrom. Rev. 21 (2002) 388.
[22] K. Tang, D.J. Fu, D. Julien, A. Braun, C.R. Cantor, H. Köster, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 10016.
[23] X. Chen, L. Levine, P.Y. Kwok, Genome Res. 9 (1999) 492.
[24] P.Y. Kwok, Hum. Mutat. 19 (2002) 315.
[25] M. Ronaghi, S. Karamohamed, B. Pettersson, M. Uhlen, P. Nyren, Anal. Biochem. 242 (1996) 84.
[26] T. Langaee, M. Ronaghi, Mutat. Res. 573 (2005) 96.
[27] F. Patolsky, A. Lichtenstein, I. Willner, J. Am. Chem. Soc. 123 (2001) 5194.
[28] M. Cho, S. Lee, S.-Y. Han, J.-Y. Park, M.A. Rahman, Y.-B. Shim, C. Ban, Nucleic Acids Res. 34 (2006) e75.
[29] J.F. Liu, D. Xing, X.Y. Shen, D.B. Zhu, Biosens. Bioelectron. 20 (2004) 436.
[30] D.B. Zhu, D. Xing, X.Y. Shen, J.F. Liu, Biochem. Biophys. Res. Commun. 324 (2004) 964.
[31] J.H. Kenten, J. Casadei, J. Link, S. Lupold, J. Willey, M. Powell, Clin. Chem. 37 (1991) 1626.
[32] X.M. Zhou, D. Xing, D.B. Zhu, L. Jia, Electrochem. Commun. 10 (2008) 564.
[33] W.J. Miao, Chem. Rev. 108 (2008) 2506.
[34] E. Terpetschnig, H. Szmacinski, H. Malak, J.R. Lakowicz, Biophys. J. 68 (1995) 342.
[35] Y.H. Pei, D. Xing, X.J. Gao, L. Liu, T.S. Chen, Apoptosis 12 (2007) 1681.
[36] D.B. Zhu, D. Xing, X.Y. Shen, J.F. Liu, Q. Chen, Biosens. Bioelectron. 20 (2004) 448.

[37] P. Nilsson, B. Persson, M. Uhlen, P.-A. Nygren, Anal. Biochem. 224 (1995) 400.
[38] H. Xu, H.P. Wu, F. Huang, S.P. Song, W.X. Li, Y. Cao, C.H. Fan, Nucleic Acids Res. 33 (2005) e83.
[39] N.R. Markham, M. Zuker, Nucleic Acids Res. 33 (2005) 577.
[40] S. Gudibande, J.H. Kenten, J. Link, Mol. Cell Probes 6 (1992) 495.
[41] C.V. de Moura Gallo, E.S. Azevedo, G. Mendonca, E. de Moraes, M. Olivier, P. Hainaut, Mutat. Res. 589 (2005) 192.