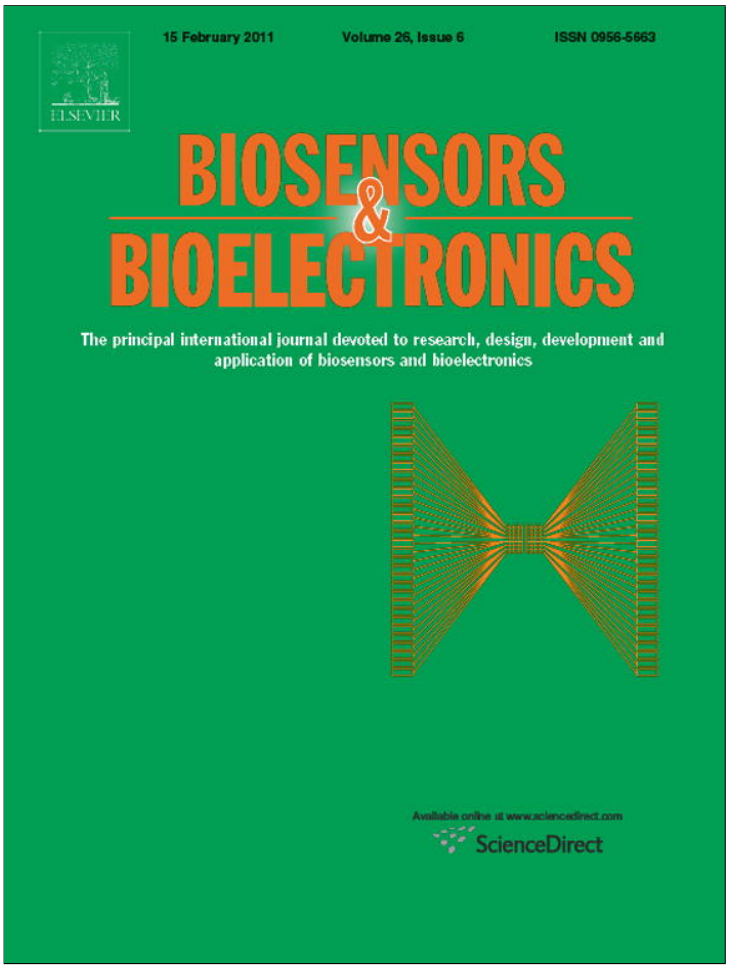

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Sensitive and isothermal electrochemiluminescence gene-sensing of *Listeria monocytogenes* with hyperbranching rolling circle amplification technology

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ABSTRACT

Listeria monocytogenes (*L. monocytogenes*) is one of the most problematic human pathogens, as it is mainly transmitted through the food chain and cause listeriosis. Thus, specific and sensitive detection of *L. monocytogenes* is required to ensure food safety. In this study, we proposed a method using hyperbranching rolling circle amplification (HRCA) combined with magnetic beads based electrochemiluminescence (ECL) to offer an isothermal, highly sensitive and specific assay for the detection of *L. monocytogenes*. At first, a linear padlock probe was designed to target a specific sequence in the *hly* gene which is specific to *L. monocytogenes* and then ligated by Taq DNA ligase. After ligation and digestion, further amplification by HRCA with a biotiny labeled primer and a tris (bipyridine) ruthenium (TBR) labeled primer was performed. The resulting HRCA products were then captured onto streptavidin-coated paramagnetic beads and were analyzed by magnetic beads based ECL platform to confirm the presence of targets. Through this approach, as low as 10 aM synthetic *hly* gene targets and about 0.0002 ng/μl of genomic DNA from *L. monocytogenes* can be detected, the ability to detect at such ultratrace levels could be attributed to the powerful amplification of HRCA and the high sensitivity of current magnetic bead based ECL detection platform.

1. Introduction

Listeria monocytogenes (*L. monocytogenes*) is an opportunistic intracellular foodborne pathogen that has been responsible for outbreaks of listeriosis. From 1979 to 1999 in the United States and Canada, there were six major outbreaks of listeriosis (Churchill et al., 2006). The most vulnerable groups include pregnant women, the elderly, newborns and immuno compromised patients. The bacteria are transmitted through ready-to-eat food. Infection with *L. monocytogenes* can result in influenza-like symptoms, meningitis, encephalitis, septicaemia, central nervous system damage and, in pregnant women, foetal infection and/or abortion. In fact, *L. monocytogenes* has a high fatality rate approaching 30%, which far exceeds other foodborne pathogens (Donnelly, 2001; Lunden et al., 2004; McLauchlin et al., 2004; Liu, 2006; Vanegas et al., 2009). Therefore, it is critical to develop an inexpensive, specific and highly sensitive method to detect *L. monocytogenes*.

Conventional methods for the identification of pathogen bacteria are culture-based and require individual biochemical confirmation of the species in a number of isolated colonies, these methods are sensitive and inexpensive but they are labor-intensive, time consuming, and not always reliable (Donnelly, 1999). As a result, much effort has been devoted towards the design of methods for efficient detection of pathogens. For instance, the immunological based biosensors including immunoblotting or enzyme-linked immunosorbent assay (ELISA) (Rajashekara et al., 1999; Valdivieso-Garcia et al., 2001), electrochemical assays (Gehring et al., 1996; Che et al., 2000), and array based biosensors (Zhou et al., 1998; Seo et al., 1999). These methods have overcome these drawbacks but they are not always the perfect choices because of the lack of sensitivity and specificity (Ahn and Walt, 2005). With the advent of polymerase chain reaction (PCR)-based methods, the field of bacteria detection has been revolutionized due to its high sensitivity and specificity (Klein, 2002; Rodriguez-Lazaro et al., 2004). However, PCR is considered to be complicated for requiring complicated thermal cycling steps and thus need sophisticated and expensive equipment. What's more, a number of drawbacks about PCR such as the nonspecific amplification generated by conventional PCR or cannot be configured for on-chip amplification also have limited its routine use in many laboratories (Nallur et al., 2001; Li et al., 2005; Wang et al., 2005). Recent developments in isothermal amplification techniques offer a wide variety of signal-amplification tools, among which rolling circle amplification (RCA) is a representative technology due to its simplicity and high efficiency (Baner et al., 1998; Huang et al., 2007; Zhang et al., 2009; Cheng et al., 2010; Su et al., 2010). However, to be useful in solution-based diagnostic

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and to find a wider application, the sensitivity must be markedly enhanced (Thomas et al., 1999). Thus, the development of a highly sensitive, specific, and low-cost method for food hygiene inspection remains a challenge.

Recently, the invention of hyperbranched rolling circle amplification (HRCA) has gained considerable attention as its isothermal and exponential amplification capacity (Lizardi et al., 1998). HRCA initiates from hybridization of a linear padlock probe to a sample DNA and then ligated by DNA ligase to form a circular probe. The resulting circular probe then can serve as the template for HRCA to proceed as a turn-by-turn cascade of multiple hybridization, primer extension, and strand displacement involving two primers (Zhang et al., 2001; Cheng et al., 2009). This complex pattern of DNA strand displacement ensures a 10^9-fold signal amplification of each circle within 90 min (Lizardi et al., 1998). Furthermore, the advantages of avoiding complicated thermocycling and high sensitivity make the technique find its wide applications in DNA (Zhang et al., 2002; Tong et al., 2007; Kaocharoen et al., 2008), RNA (Wang et al., 2005; Cheng et al., 2009), and protein detection (Zhang and Liu, 2003). Currently, electrophoresis and fluorescence based methods are dominantly available detection technologies for HRCA product detection. However, electrophoresis is generally considered as inefficient tool due to its labor-intensive characteristic. As to fluorescence based methods, the high background resulted from free fluorescent probes or dyes as well as the reflection and scattering of light source is a limitation for further improving sensitivity of fluorescent based methodologies (Guo et al., 2009).

Electrochemiluminescence (ECL) can be considered as an important and powerful tool in analytical and clinical application with high sensitivity, excellent specificity, and low cost (Blackburn et al., 1991; Menking et al., 1995; Yu and Bruno, 1996; Chen et al., 1997). Specifically, the considerable advances have been made in DNA biosensors using tris (2,2′-bipyridyl) ruthenium (TBR) with tripropylamine (TPA) ECL detection protocol. Furthermore, magnetic bead based ECL method with TBR and TPA reaction has been used widely in clinical diagnostics, life sciences research, environmental assays such as food and water testing and biological threat agents (Richter, 2004; Miao and Bard, 2004; Miao, 2008; Zhou et al., 2009). The use of surface-functionalized magnetic beads serves not only for a rapid and efficient separation tool but also for a high throughput platform for detection. It has proven to be as a versatile tool in the separation and enrichment of nucleic acids, proteins, and other biomacromolecules (Olsvik et al., 1994; Yan et al., 2004; Pyati and Richter, 2007; Lin et al., 2007).

In this present study, we describe a magnetic beads based ECL HRCA method for *L. monocytogenes* detection with high sensitivity and specificity. The *hly* gene that encodes a cholesterol-dependent cytolysin responsible for clinical symptoms and is highly conserved in *L. monocytogenes* was chosen as a target for specific detection (Churchill et al., 2006). The proposed method takes advantage of the efficient exponential amplification of HRCA and intrinsically high sensitivity and low cost of ECL. Besides, the streptavidin-coated magnetic beads used here served as both the separation tool and the immobilization matrix. Thus the ECL HRCA product can be captured onto streptavidin-coated paramagnetic beads through biotin-streptavidin interaction and ECL detection can be executed in situ at the surface of a platinum electrode. Through this method, a detection limit of synthesized *hly* gene target was 10 aM, which exhibits excellent sensitivity for DNA analysis. After that, we applied the method to detect genomic DNA from *L. monocytogenes* under optimized contidion. Results showed that 0.0002 ng/μl of genomic DNA from *L. monocytogenes* can be detected and other kinds of bacteria were not detected. We anticipate that the proposed magnetic beads based ECL HRCA strategy will find numerous applications in food safety field.

2. Experimental

2.1. Apparatus

A custom-built ECL detection system has been described in our previous research (Zhu et al., 2004; Duan et al., 2010). An electrochemical reaction cell with a working electrode (platinum), a counter electrode (platinum) and a reference electrode (Ag/AgCl) comprises the heart of the instrument. An optical fiber-bundle is used to receive the light emit from the ECL reaction and conduct it to an ultra high-sensitive single photon counting module (PMT, MP-962, PerkinElmer, Wiesbaden, Germany). To collect the magnetic captured products to the proper locations for ECL detection, a magnet is placed closely under the working electrode. Besides, a potentiostat (Fujian Sanming HDV-7C), a multi-function acquisition card (Advantech PCL-836), a computer and LabView software are also the necessary components of our equipment.

2.2. Materials

The Taq DNA ligase, Bst DNA polymerase large fragment and their corresponding buffer are purchased from New England Biolabs (NEB), the Exonuclease I, Exonuclease III, Bsp1286 I, EcoT14 I and their corresponding buffer are from Takara Bio (Shiga, Japan), *L. monocytogenes* (CMCC54007), *Listeria innocua* (GIM1.230), *Salmonella enterica* (CMCC50040) and *Escherichia coli O157:H7* (GW1.2020) are obtained from Guangzhou Institute of Microbiology, China. The deoxynucleotide solution mixture dNTPs is ordered from MBI (Fermentas, USA). All oligonucleotides used in our research are synthesized and purified by HPLC at Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (SSBE) (Table 1). The padlock probes are chemically 5′-phosphorylated, the HRCA primer 1 are labeled with biotin and the HRCA primer 2 are tagged with the Ru $(bpy)_3^{2+}$-NHS. TPA and the chemicals to synthesize the Ru $(bpy)_3^{2+}$-NHS are the products of Sigma (St. Louis, MO, USA). Streptavidin-coated magnetic beads (2.8 μm in diameter) are purchased from Dynal Biotech (Lake Success, NY, USA). Other chemicals employed are of analytical reagent grade and are used as received. The high-purity deionized water (resistence >18 MΩ cm) is used throughout.

2.3. Genomic DNA extraction

L. monocytogenes was grown in brain heart infusion broth at 37 °C with shaking overnight. The bacteria DNA isolated from 1 ml cultures was extracted according to the manufacturer's protocol from the TIANamp Bacteria DNA Kit which was purchased from Tiangen biotech (Beijing) Co. Ltd. (Beijing, China) and quantified by measuring the optical density at 260 nm with a spectrophotometer (Eppendorf BioPhotometer, AG 22331 Hamburg, Germany).

2.4. Ligation and exonuclease treatment

Genomic DNA was fragmented by digestion using Bsp1286 I and EcoT14 I for 60 min and then used as template. Hybridization of target sequences with the padlock probes was carried out in 20 μl ligation system containing 20 mM Tris–HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, 1 mM nicotinamide adenosine dinucleotides 0.1% Triton X-100, and 12 U of Taq DNA ligase, 100 nM linear padlock probes, different concentration of *hly* gene or genomic DNA template. The mixture was incubated at 60 °C for 60 min, and 95 °C for 10 min to inactivate the enzyme. After that, 10 μl ligation products were added to 10 μl exonuclease mixture containing 67 mM glycine-KOH (PH 9.5), 1 mM DTT, 6.7 mM $MgCl_2$, 10 U Exonuclease I and 20 U Exonu-

Table 1
Italics indicate sequences complementary to *hly* genes of pathogenic *Listeria monocytogenes*; bold italics indicate the binding region for RAM primer 1; bold indicate the binding region for RAM primer 2. As to the hly target, italics indicates the C-probe binding region; the vertical bar indicates ligation site of the 5′ and 3′ end of the C-probe.

DNA	Sequence (5′–3′)
Padlock probe	Phosphate-*GCGTCTTAGGACTTGCAGGCGGG**ATTAGGTTACTGCGATTA** **GCACAAG**C**ACCAAGAGCAACTACACGAATTC**CTGTTTCTTTTCGATTG*
RAM primer 1	Bio-CTTGTGCTAATCGCAGTAACCTAAT
RAM primer 2	TBR-ACCAAGAGCAACTACACGAATTC
Hly target	TCT*CCGCCTGCAAGTCCTAAGACGC*|*CAATCGAAAAGAAACA*CGC
PCR primer 1	GCCTGCAAGTCCTAAGACGCCAATC
PCR primer 2	CTTGCAACTGCTCTTTAGTAACAGC

clease III. The mixture was incubated at 37 °C for 2 h, followed by inactivation at 95 °C for 10 min.

2.5. HRCA reaction

After ligation and circularization of the probe, a followed step was performed to amplify the circular probe by using Bst DNA polymerase with strand displacing activity. HRCA reactions were performed in a 60 μl volume containing 8 U Bst DNA polymerase, 400 μM deoxynucleoside triphosphate mix, 1 μM of each labeled HRCA primer, and 20 μl of the post-digestion mixture. Circularized probe signals were amplified by incubation at 63 °C in the presence of 1 μg bacteriophage T4 Gene 32 protein (NEB) and 5% DMSO for 60 min.

2.6. PCR amplification

The extracted DNA was tested by PCR to confirm the presence of *hly* gene. PCR was carried out in a 20 μl reaction mixture composed of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM concentrations of each deoxynucleoside triphosphate, 250 nM of each primer (Table 1), 0.5 unit of Taq DNA polymerase (TaKaRa), and 2 μl of bacterial lysate. The reaction was performed in a PTC – 100 thermocycler (MJ Research) for 30 cycles. The cycling procedures were set as follows: an initial step of denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, anneal at 61.3 °C for 1 min and elongation at 72 °C for 1 min; final extension at 72 °C for 8 min. Five microliters of PCR products was analyzed by gelelectrophoresis using a 2.0% agarose gel containing 0.5 μl/ml of GoldView™ as fluorescence dye. The running conditions were constant voltage at 100 V. After electrophoresis, which occupied about 30 min, the relative amounts of PCR products were analyzed by image analysis software (Quantity One™, Bio-Rad, CA, USA). The DL 2000 DNA markers, which contain 2000, 1000, 750, 500, 250, and 100 bp DNA fragments, were used as standards for the evaluation of the gels. The expected PCR product is 706 bp for *hly* gene.

2.7. ECL detection

For analyzing the samples, 60 μl HRCA products and 100 μg streptavidin coated beads were added to 50 μl bind buffer (10 mM TE, 500 mM NaCl, PH 7.4), and then the mixture was incubated at 37 °C for 30 min. The reaction mixture was separated by using magnetic racks (Dynal, mpc-s) and washed twice with bind buffer to remove the unbound TBR-labeled HRCA primer. The remained target analytes bound on the magnetic beads were resuspended in 100 μl ECL assay buffer (200 mM phosphate, 50 μM NaCl, 7 mM NaN_3, 0.8 μM Triton X-100, 0.4 mM Tween 20, 100 mM TPA, pH 8.0). After washing and removing the supernatant, the sample was transferred to the flow ECL detection 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 electrodes and the photon signal was measured by PMT. At last, computer read the ECL signals by labview software.

The threshold values to define a specific sample for detecting is calculated based on formula (1), the $V_{negative}$ and $V_{stdev(neg)}$ represent the averages and standard deviations of the ECL reading from the negative control samples. A sample with an ECL value higher than the threshold values (V) is considered to be positive.

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

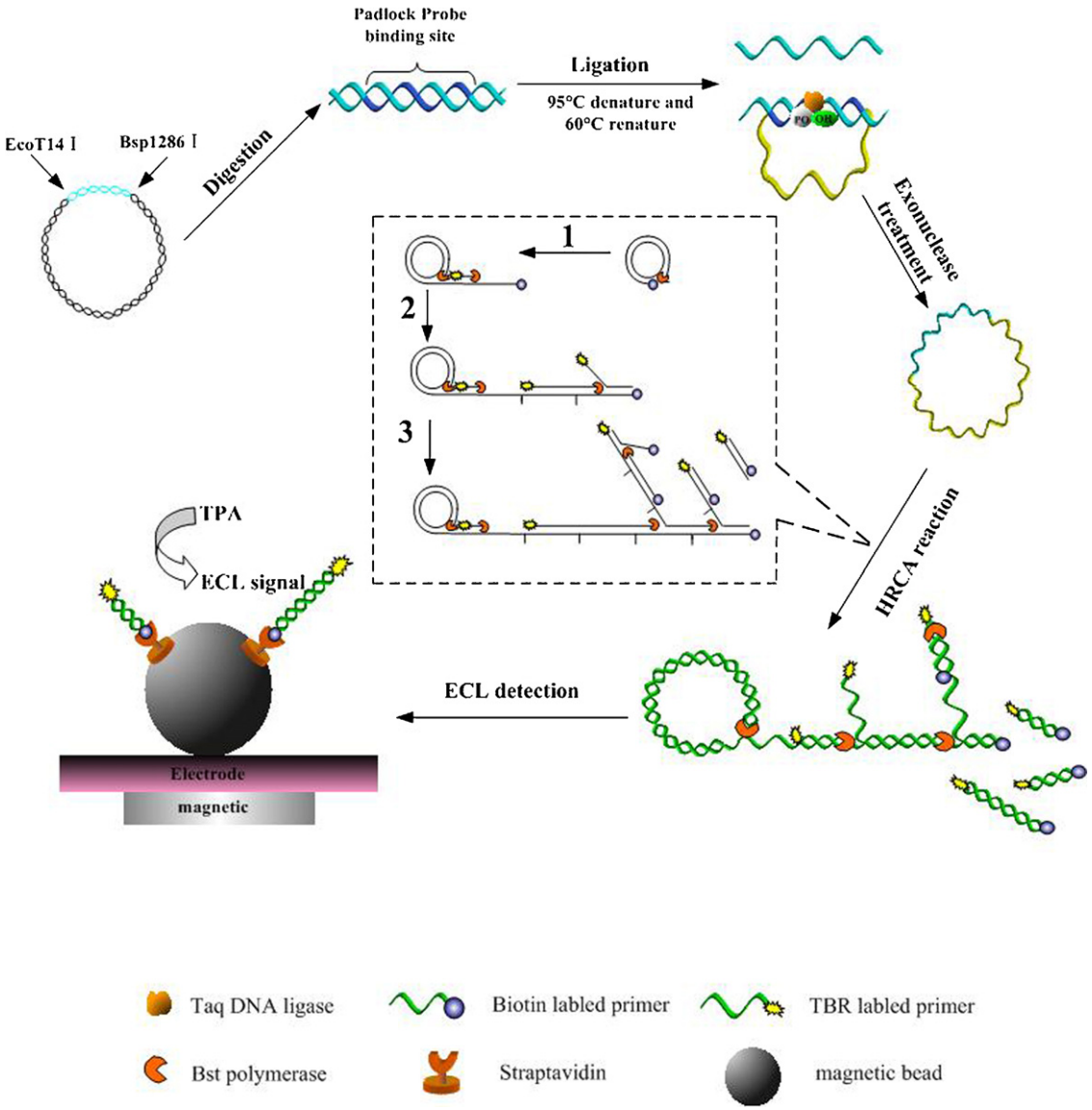
3. Results and discussion

3.1. The principle of magnetic beads based ECL HRCA for detecting target DNA

The basic principle of the proposed method is illustrated in Scheme 1. In this method, the padlock probe is 89 nucleotides (nt) in length, consisting of four distinct regions: a special region hybridized with biotinylated primer, a region that is the same as the TBR-labeled primer, and two target complementary sequences located at the 5′ and 3′ termini. Only when the two termini of padlock probe are perfectly hybridized to the complementary target sequences, the linear padlock probe can be specifically ligated by Taq DNA ligase. In order to reduce subsequent ligation-independent amplification, un-reacted padlock and excess oligonucleotides in the ligation products were digested by Exonucleases I and III (Marianna et al., 2005). Following the digestion, the biotinylated primers which hybridized to a special region of resulting circular padlock probes are extended isothermally at its 3′ end by Bst DNA polymerase to generate multimeric single-stranded DNA (ssDNA). This multimeric ssDNA can serve as the template for the TBR-labeled primer binding and then be further amplified by Bst DNA polymerase through TBR-labeled primer extension and downstream DNA displacement; this displaced strand will in turn contain multiple binding sites for biotinylabled primer. Thus, strand displacement process generates a continuously expanding pattern of DNA branches connected to the original circle and a discrete set of free double-tagged DNA fragments comprising double-stranded pieces of the unit length of a circle and mutiples thereof. The flow chart of HRCA is showed in the inset. After the HRCA reaction, the products are concentrated by streptavidin coated magnetic beads through the highly selective biotin–streptavidin linkage. The unextended TBR-labeled primer can be easily washed off with the assist of magnetic separator. Thus, only the doubled-tagged amplicon can be detected in the detection cell. Then 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 magnetic beads based ECL HRCA experiment condition

The success of the current assay is basically dependent on ligation reaction. In order to minimize the secondary structure



Scheme 1. The principle of magnetic beads based ECL HRCA assay for *Listeria monocytogenes* detection. (A) Genomic DNA digestion and padlock probe circularization. (B) HRCA reaction with a biotiny labled primer and a tris (bipyridine) ruthenium (TBR) labeled primer. (C) Electrochemiluminescent (ECL) detection.

presence in padlock probe and assure the high specificity, a suitable hybridization temperature should be determined. Herein, a series of hybridization temperatures varied from 45 °C to 70 °C with an interval of 5 °C were evaluated. In addition, the concentration of padlock probe was set at 100 nM for all experiments in order to achieve full hybridization to the target strands whose concentration is set as 1 fM. As presented in Fig. 1A, the ECL intensity peak was observed at around 60 °C, while the ECL value of control was always low and changed scarcely. Hence, 60 °C was chosen for hybridization of the padlock probe and target in all the following experiments.

For the purpose of obtaining the high efficiency of ECL detection, the amplification duration and ECL intensity were evaluated. The amplification duration was varied from 0 to 105 min with an interval of 15 min increments. Fig. 1B indicates that the ECL readout increased linearly with the HRCA reaction time up to 60 min. However, the signal exhibited non-linearly increase when the reaction duration went beyond 60 min. It may be attributed to the fact that the activity of the Bst DNA polymerase had decreased. In parallel assays with the control, weak ECL signal was always showed regardless of the amplification time. Taking quantitative measurement and reduction of sensing time into consideration, we selected 60 min as the optimal amplification time.

In the proposed method, the quantity of magnetic beads is a vital factor to influence the ECL intensity. The binding of biotin-labeled DNA primer and the surface of streptavidin-coated beads is quick, reliable and strong ($K_d = 10^{-15}$). This feature makes the unlinked DNA fragments are then be washed away easily and do not affect the HRCA products. In addition, the magnetic beads can condense the products thus form a $Ru(bpy)_3^{2+}$ domain by a magnet and result in higher sensitivity. However, when the excessive beads are present in the ECL reaction system, the electrode would be overpopulated by the adsorbed beads to reduce the reaction of TPA and TBR. Consequently, appropriate amount of magnetic beads was required for capturing the entire HRCA products. Fig. 1C demonstrates the effect of the different quantity of magnetic beads from 40 μg to 160 μg on the ECL intensity. It was observed that the ECL response was firstly increased and then decreased, the maximum ECL intensity was observed at 100 μg. On the contrary, the ECL signal obtained from control was always low. Therefore, 100 μg beads were added to each detection system throughout the experiments.

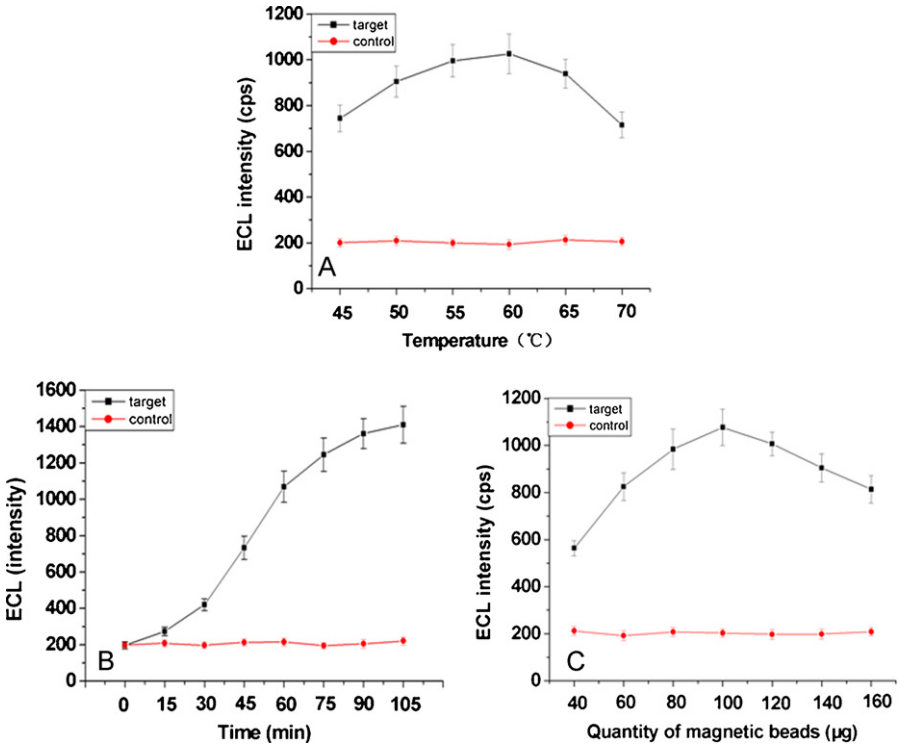


Fig. 1. Optimization of HRCA experiment conditions: (A) Hybridization temperature-dependent ECL intensity changes. (B) ECL intensity of different amplification time. (C) The effect of the quantity of magnetic beads on the ECL intensity. All reactions are carried out as described in Section 2. Error bars show standard deviation which was determined by at least three replicates.

3.3. The specificity of current assay

Under the optimized experiment condition, we did the experiment with a DNA sequence homologous to part of the target gene *hly* whose concentration is 100 nM to evaluate the feasibility of the method. Fig. 2A displays the ECL results obtained from target-free control (blank) and 100 nM synthetic *hly* gene target. It was observed that the average ECL signal obtained from analysis of *hly* gene was 3145.4 ± 266.5 counts per second (cps) which was significantly higher than that of control (207.1 ± 18.7 cps), demonstrating that the proposed method can be used to distinguish the *hly* gene from control markedly.

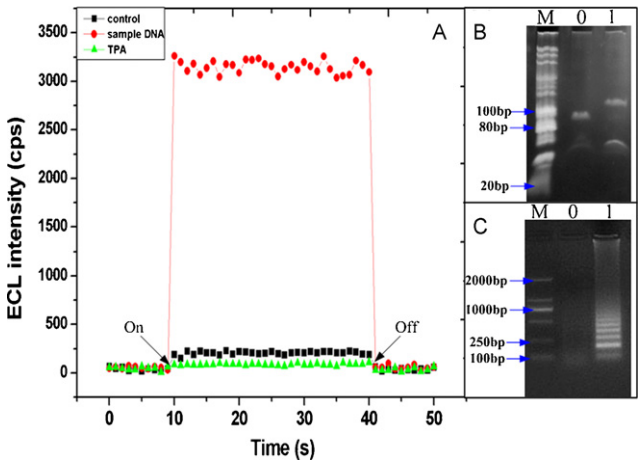


Fig. 2. Specificity evaluation of ECL HRCA method. (A) ECL intensities corresponding to *hly* gene-free control, 100 nM *hly* gene and TPA. On: potentiostat on. Off: potentiostat off. (B) Electrophoretic identification of the enzymatic ligation reaction. The ligation products are separated by 10% polyacrylamide gel electrophoresis containing 8 M urea, and DNA is screened with a standard silver-staining method. The 20 bp, 80 bp, 100 bp ladder is indicated in lane M. Lanes 0, 1 represent the ligation products from target-free control and target, respectively. (C) HRCA products are electrophoresed in a 1% agarose gel stained with GoldView™ dye. The DNA ladder is indicated in lane M. Lanes 0, 1 represent the HRCA products from target-free control and target, respectively.

In order to further verify the specificity of the ligation reaction, the resulting products of the ligation were analyzed by 10% denaturing polyacrylamide gel electrophoresis stained with a standard silver-staining method. As can be seen in Fig. 2B, the M lane represents 20 bp DNA ladder, the lanes 0, 1 represent the ligated product from blank and special target, respectively. It is obvious that in lane 1 the product of ligation moved slower than the linear padlock, which indicates that the linear padlock was ligated into a circular form in the presence of target. Then, the linear oligonucleotides in the ligation products were digested by Exonuclease I and Exonuclease III. Exonucleolysis is required to remove the unreacted probes so that they could not lead to ligation-independent amplification, and then reduce background signal (Hafner et al., 2001). HRCA reactions were performed using the Bst DNA polymerase in the presence of biotinylabeled primer and TBR-labeled primer at 63 °C. After that, the resulting products of HRCA were electrophoresed in a 1% agarose gel. As shown in Fig. 2C, the M lane represents DNA ladder, the lanes 0 and 1 represent the amplified product derived from blank and special target, respectively. There are obvious ladder-type bands in lane 1 (plus target), which reveals that a discrete set of concatemeric dsDNA fragments are formed. In contrary, no ladder type bands were found in lane 0 (control). It indicated that the discrete set of concatemeric dsDNA fragments could be produced with a high specificity. The results of gel electrophoresis were consistent with the results of ECL detection.

3.4. The sensitivity of special target combining ECL detection

To evaluate the sensitivity of the magnetic beads based ECL HRCA method, different concentration of *hly* gene segments were tested with invariable 100 nM padlock probes, and then the lig-

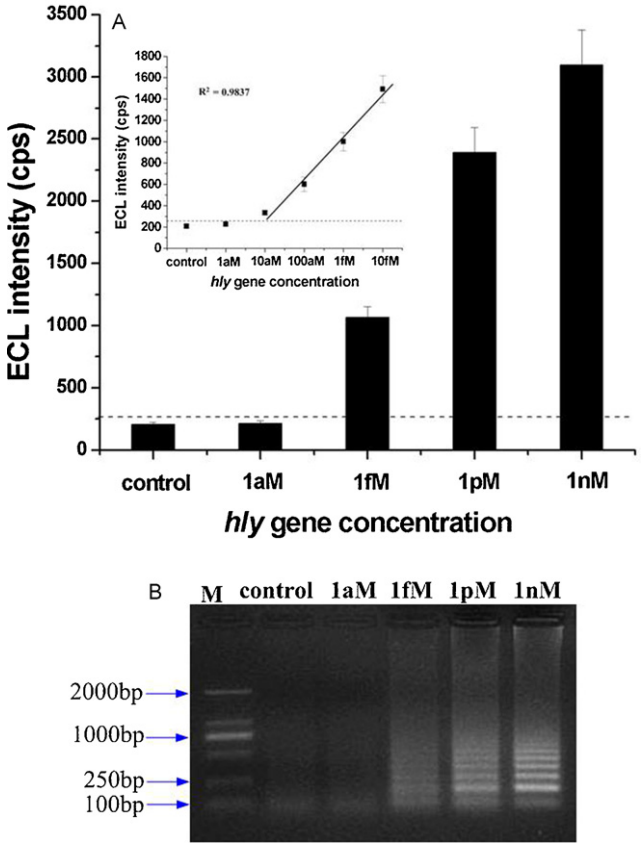


Fig. 3. (A) The sensitivity assessment of ECL HRCA method. The dashed line represents the threshold value calculated based on formula (1). (B) Analysis of HRCA reaction from different concentration of *hly* gene on electrophoresis.

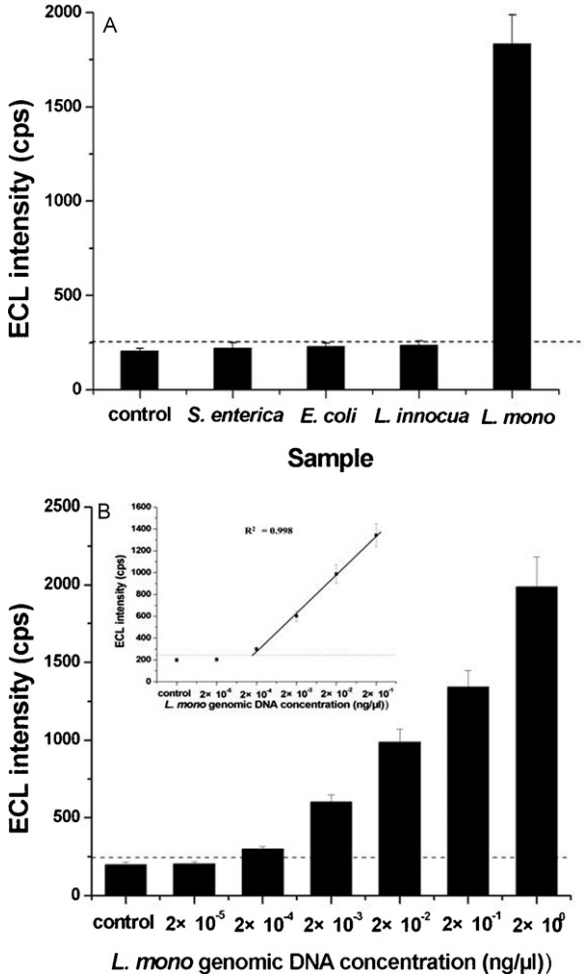


Fig. 4. (A) Genomic DNA from *S. enterica*, *E. coli*, *L. inno* and *L. mono* were analyzed by magnetic beads based ECL method. (B) The sensitivity assessment of ECL HRCA method applied to genomic DNA detection.

ated circular templates were amplified. In Fig. 3A, the ECL intensity was plotted against the target concentration. The response signal increases with the increase of target concentration. The response of ECL intensity in the inset picture show that the detection of as little as 10 aM can be achieved. At this concentration, the ECL value of *hly* gene and control was 332.8 ± 25.5 cps and 206.8 ± 15.7 cps, respectively. According to the formula (1), the threshold value was set as 253.7 cps, indicating the special target could be discriminated apparently. To investigate the precision of the proposed assay, relative standard deviation (RSD) was determined by measuring ECL signal in different concentration with six replicates. The RSD of ECL intensities are less than 10% at the concentration of 1 fM, 1 pM, and 1 nM, which suggesting an acceptable reproducibility. Besides, the data shows a good linear correlation between ECL signals and the different concentration of target from 10 aM to 10 fM. The results for determining the sensitivity well demonstrates the extraordinary capability of our magnetic beads based ECL HRCA assay in detecting *hly* gene. The high sensitivity is due to the powerful amplification ability of HRCA and the increased sensitivity utilizing magnetic beads combined with ECL.

To validate whether the different ECL intensity comes from the effect of concentration of *hly* gene samples and determine the HRCA efficiency, 1% agarose gel electrophoresis analysis for HRCA products from 1 aM, 1 fM, 1 pM, 1 nM targets was performed. As can be seen in Fig. 3B, the ladder-type bands were observed at the concentration of 1 fM, 1 pM, and 1 nM, but not in the control samples and the 1 aM targets. Besides, there was an increase of HRCA products with the increase of targets. These results indicated that the ECL intensity comes from the effect of concentration of *hly* gene samples and the proposed method offers well-defined concentration dependence. The ladder of products is consistent with this mechanism of the geometric HRCA, which proceeds as a turn-by-turn cascade of multiple-hybridization, primer-extension, and strand-displacement events involving both primers. So the high sensitivity can be achieved by this proposed method.

3.5. Detection of bacterial genomic DNA

Magnetic beads based ECL HRCA was applied to detect genomic DNA from foodborne pathogen, *L. monocytogenes*. Padlock probe was designed to detect a complementary strand of its virulence gene, *hly* (GeneBank GeneID 223702383), encoding a cholesterol-dependent cytolysin, listeriolysin O (LLO) (Churchill et al., 2006). When the linear padlock probe hybridizes to the gene, the circular padlock probe can be formed to initiate HRCA. BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov) search of the 38-base target in *hly* gene showed highly specific for *L. monocytogenes*, and no exact match against *S. enterica*, *E. coli*, and *L. innocua* genome.

The 2 ng/μl of *L. monocytogenes* genomic DNA was analyzed by the proposed method under the optimized condition determined above. In addition, a restriction digestion step was conducted to prevent DNA supercoiling and decircularization of the genomic DNA during the heating step (Hill et al., 2007). As show in Fig. 4A, the ECL signal obtained from *L. monocytogenes* and control was 1832.5 ± 156.4 cps and 205.8 ± 15.01 cps, respectively. According to the formula (1), the ECL signal obtained from *L. monocytogenes*

genomic DNA is much higher than the threshold value of 251.1 cps. However, the ECL intensity emitted upon analyzing the different bacteria originated from *S. enterica*, *E. coli* and *L. innocua* are 221.4 ± 19.4 cps, 228.6 ± 17.0 cps, 235.8 ± 20.5 cps, which were all below the threshold value (251.0 cps). It is shown that the proposed method is highly selective to *L. monocytogenes* over other bacteria, which attributes to the specific padlock probe design, in which the Tm of 5′ arm is close to or above the ligation temperature and the T_m of 3′ arm is 10–15 °C below ligation temperature. Thus, the 5′ end arm serves as an anchor sequence and the 3′ end arm is an equilibrium process. According to BLAST result, the 5′ arm was selected highly specific for *L. monocytogenes*. Therefore, the presented assay can detect *L. monocytogenes* with high specificity.

To further confirm the sensitivity of the proposed method, serially dilluted genomic DNA from *L. monocytogenes* (0.0002–2 ng/μl) was analyzed under optimized condition determined above, as shown in Fig. 4B. It is shown the detection limit is down to 0.0002 ng/μl (about 1360 copies/reaction) was achieved, whose ECL intensity was 298.2 ± 18.4 cps, which is higher than threshold value (247.5 cps) according to formula (1). Linearity of ECL intensity was observed from 2×10^{-4} ng/μl to 2×10^{-1} ng/μl. When applied this method to detect *L. monocytogenes* in milk, a sensitivity of 5×10^4 CFU/ml was achieved (see Supplementary information). This sensitivity is much higher than those DNA-based detection platforms reported previously and is comparable with PCR (Dunbar et al., 2003; Baeumner et al., 2004; Liao and Ho, 2009).

The high sensitivity can be attributed to the following factors. Firstly, the Bst DNA polymerase has shown a strong and reliable ability to displace newly synthesized DNA strands under isothermal conditions, so a large number of double-tagged DNA fragments will bind with the magnetic beads. 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 biotin-labeled HRCA products can be 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 surface, resulting in a rapid detection process and a reduced detection cost.

Most conventional methods for detecting foodborne bacterial pathogens by using microbiological media to selectively grow and enumerate bacteria. The advantages of these methods are sensitive, inexpensive and provide qualitative as well as quantitative results. However, these methods are culture-dependent and the enrichment step would lasts from 24 to 48 h. Besides, preparation of media and plates, as well as colony counting and biochemical characterization of the isolated colonies is labor-intensive process (De Boer and Beumer, 1999). Immunoassay-based methods for detection of bacteria are fast (usually in few hours) and relatively inexpensive. But there are still many problems when applying it in practical detection. Such as the low sensitivity of the assays, low affinity of the antibody to the pathogen or potential interference from contaminants (Churchill et al., 2006). Therefore, one of the best ways to detect bacteria pathogen is using nucleic acid-based methods to ensure high specificity. PCR is the most widespread technique in detecting bacteria due to its high sensitivity and specificity. Dunbar et al. explored multiplex PCR with microsphere sorting to detect bacteria and reach the sensitivity of 10^3–10^5 genome copies per reaction by pure culture without preenrichment in 4 h (Dunbar et al., 2003). The method brought up by Koo and Jaykus provides a detection limit of about 500 cfu/ml in pure culture in 2.5 h and 10^3–10^4 cfu/ml organisms in skim milk without preenrichment by the use of FRET-PCR (Koo and Jaykus, 2003). However, PCR requires high-precision temperature cycling and thus need expensive thermo cycler, what's more, problems with the in situ applications have hindered its routine use in many laboratories. Thus there is a strongly request to find a robust isothermal alternatives (Vadim, 2002). Barry and Stephen compared various isothermal alternatives and revealed that RCA could be the unique one and would be the best choice for DNA target amplification other various fields (Barry and Stephen, 2001). In this work, we combined the magnetic beads based ECL and HRCA to have achieved the sensitivity of 1360 genome copies per reaction in pure culture and 5×10^4 cfu/ml when detecting *L. monocytogenes* in milk, which is comparable to many PCR based method. Besides, all the detection process can be complished in 5 h without preenrichment. The sensitivity of magnetic beads based ECL HRCA is also comparable with real time HRCA (Yao et al., 2009). However, the cost is much lower and the ECL apparatus used in this study is more cost-effective than the real time HRCA method. Considering these merits, we anticipate that our method will find numerous applications in routine food safety monitoring.

4. Conclusions

Magnetic beads based ECL HRCA method is useful to detect *hly* gene in *L. monocytogenes*. The assay takes advantage of the high amplification efficiency of HRCA and the intrinsically high sensitivity of ECL, leading to a limit of detection of 0.0002 ng/μl of *L. monocytogenes* genomic DNA with high specificity. This method demonstrates several analytical advantages. Firstly, the ECL HRCA method presented here is sensitive, specific, and robust, which has advantages over the PCR method, namely, reaction simplicity, high specificity, and high amplification efficiency. Moreover, since HRCA can avoid complicated thermal cycling steps (an isothermal reaction at 37 °C), heating in a short of time (60 min) using a simple incubator such as a water bath or a block heater is sufficient to amplify DNA to detectable levels. Furthermore, the proposed method can detect target by only measuring the ECL intensity at the end of reaction, so this technique entails no specialized devices besides our custom-built instrument. This can also markedly reduce the cost. Last but not least, combining with the magnetic beads based ECL technique, the proposed assay can be easily extended to a high-throughput, multipurpose and automatic screening format. This capability is indeed a desired feature for detection of pathogens. Eventually, we anticipate that this method has enormous potential for application of a variety of biorelated markers detection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.11.034.

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