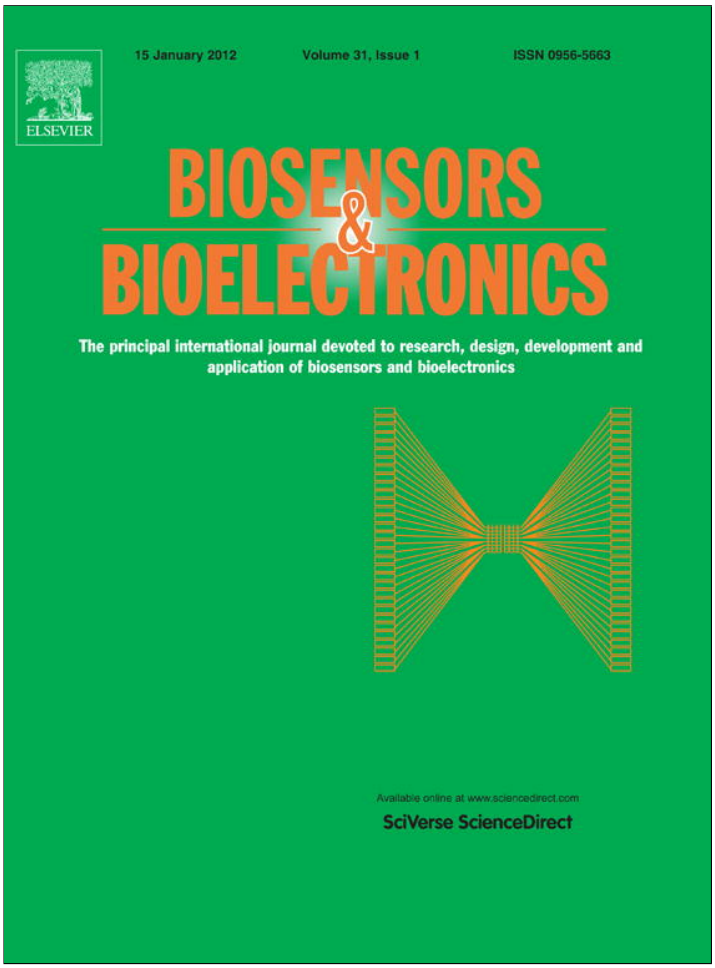

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Nano-magnetic primer based electrochemiluminescence-polymerase chain reaction (NMPE-PCR) assay

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ABSTRACT

Here we have developed a novel nano-magnetic primer based electrochemiluminescence-polymerase chain reaction (NMPE-PCR) strategy for detection of genome. The key idea of this method is integrating the two in situ processes: PCR on the surface of magnetic nanoparticles (MNPs) and magnetic beads based ECL readout platform, to avoid some laborious manual operations and achieve rapid yet sensitive detection. At first, the approach employs a pair of functional primers for amplification: one is tris-(2,2′-bipyridyl) ruthenium (TBR) labeled primer; the other one is nano-magnetic primer which is prepared by attaching the primer to the surfaces of MNPs. With the presence of DNA analyte and PCR mixture, the TBR labeled products are directly loaded and enriched on the surface of MNPs during PCR cycling. Then the MNPs–TBR complexes can be analyzed by a magnetic ECL platform without any post-modification or post-incubation. Finally, we used *Listeria monocytogenes* as the target to examine these desirable properties of this assay, reaching a detection limit of 500 fg/μL for genome in 1 h. The proposed study has provided the evidence as a proof-of-concept, thus having potential for development of automatic mode for detection of specific gene.

1. Introduction

DNA and RNA analysis, which aims at monitoring sequence variations or measuring copy numbers of target sequences, must be sufficiently sensitive and specific to detect small numbers of target sequences from complex background (Check, 2005; Cheng et al., 2009). Owing to the exponential ability of amplification and specific design of primers, the polymerase chain reaction (PCR) revolutionized molecular genetics, and is considered as the central technology in fundamental genetic research (Ding and Cantor, 2003; Lu et al., 2004; Fan and Hu, 2005). However, the conventional PCR is often followed by time-consuming electrophoresis detection through distinguishing the band pattern by eyes. The inevitable weaknesses of this gel-based method include low sensitivity and need to use hazardous materials such as radioactive isotopes or ethidium bromide (EB) (Chen and Viola, 1991). To overcome these limitations, there is increasing interest in developing new methods for DNA detection by scientific society (Li et al., 2005; Defever et al., 2009; Gorin et al., 2009; Lu et al., 2010). One popular methodology is electrochemiluminescence (ECL), which provides reliable, sensitive, and inexpensive approach to detect DNA (Leland and Powell, 1990; Richter, 2004; Marquette and Blum, 2008; Miao, 2008). ECL is a general term used to describe an electron-transfer reaction or mechanism on the vicinity of an electrode. The light-emitting species are produced by reaction between $Ru(bpy)_3^{2+}$ (TBR) and tripropylamine (TPA), which are the most common ECL luminophore and the most efficient known co-reactant (Deaver, 1995). Since Kenten first used ECL in DNA probe assays, the reliable technology has been expanded to most areas of bio-related analysis (Kenten et al., 1991; Zhou et al., 2009; Duan et al., 2010; Su et al., 2010).

As we know, the surface-functionalized magnetic beads have been studied extensively for cell purification, immunoassay, drug delivery due to its ability to selectively bind low-abundance target analytes (DNA, bacteria, protein). Magnetic beads serve not only as versatile tool in the separation and enrichment of biomolecules but also as high throughput platform for detection (Thaxton et al., 2005; Guan et al., 2008; Willner et al., 2008; Jang et al., 2009; Liu et al., 2009; Nie et al., 2009; Ding et al., 2010; Xiang et al., 2011). Inspired by these advantages, we and other groups have developed magnetic beads based ECL-PCR method (Liu et al., 2004; Zhu et al., 2004; Wei et al., 2010; Zhu et al., 2010). The developed electrochemical assay does not require modification of electrode for biomolecules capturing, thus the re-use of electrode after the detection is convenient. These methods have been successfully applied to DNA analysis, such as point mutations detection (Zhu et al., 2004), food safety monitoring (Wei et al., 2010), and identification of genetically modified organism (Liu et al., 2004). Nevertheless, this method is not always the optimal choice. Due to the need

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of capturing the biotinylation PCR product or hybridizing PCR product with signal probe, the assay would take at least half an hour for post-incubation and post-modification. In consideration of the time of amplification, these pretreatments of ECL detection would significantly reduce efficiency of the entire experiment, and make the methods more laborious. The additional disadvantages of this assay are the increase of the probable error as a result of adopting these manual operations. The shortcomings would limit the use of magnetic beads based ECL method for rapid detection of a mass of parallel samples at the field site or centers for disease control. Nowadays, an improved PCR technique by immobilizing the modified primers to the surfaces of micro-beads has been reported (Patolsky et al., 2003). Using the PCR mixture and temperature cycles, it could amplify plasmids on the surface of primers-functionalized beads in vitro. Very recently, James Link and his co-workers reported a new PCR which was based on the block copolymer nanobeads (Zhang et al., 2011). Compared to the micrometer of beads based PCR cycling, the amplification on nanoscale platforms could solve the considerable problem of the longer reaction time and lower efficiency of amplification induced by the settlement of particles (Kumaresan et al., 2008), and has the potential to increases the throughput of currently used bead-based DNA sequencing approaches.

Motivated by these previous theories, why not let the PCR and emission of ECL directly occur on the surface of magnetic nanoparticles (MNPs) to improve the efficiency of analysis? Herein, we first address a novel nano-magnetic primer based ECL-PCR (NMPE-PCR) detection to provide rapid, specific, yet highly sensitive approach for genome test. The key idea of the novel method is integrating the in situ nucleic acids amplification using nano-magnetic primers and in situ ECL readout on magnetic beads based detection platform. The MNPs perform two important roles in this DNA analysis: (a) immobilization of primers for in situ PCR, (b) enrichment of PCR products on electrode surface for in situ ECL detection. Therefore, the PCR products can be directly analyzed by a magnetic ECL platform without any pretreatment.

2. Materials and methods

2.1. Reagents

Carboxy coated MNPs was obtained from Allrun (100 nm in diameter, PM3-008, Shanghai, China). The Ex Taq DNA, mixture dNTPs (each 0.1 mM), their corresponding buffer and DL2000 Maker were purchased from Takara Bio (Dalian, China). *Listeria monocytogenes* (CMCC54007), *Salmonella enterica* (CMCC50040) and *Escherichia coli O157:H7* (GW1.2020) were obtained from Guangzhou Institute of Microbiology China. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-(4-morpholino) ethanesulfonic acid (MES), bovine serum albumin (BSA), TPA and the chemicals to synthesize the $Ru(bpy)_3^{2+}$-NHS were the products of Sigma (Louis, MO, USA). All oligonucleotides used in our research were synthesized and purified by HPLC at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Table S1). The TIANamp Bacteria DNA Kit was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). Other chemicals employed were all of analytical reagent grade. Water ($\geq$18.2 MΩ cm) used throughout the experiments was generated by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Apparatus and data analysis software

The ECL detection system was built by our laboratory (Liu et al., 2004; Zhu et al., 2004). The heart of the system is the electrochemical reaction cell, containing a working electrode, a counter electrode and an Ag/AgCl reference electrode. The working electrode (disk) and the counter electrode (ring) are made of platinum. The working electrode with the shape of round disk (diameter 6 mm) is at the center of the reaction cell. Magnet is located at the lower layer of working electrode to apply a magnetic field for capturing MNPs onto the surface of the working electrode, which allows effective enrich of the MNPs. An optical fiber-bundle receives the light emitted during the ECL reaction and conducts it to an ultrahigh sensitivity single photon counting module (PMT, MP-962, Perkin–Elmer, Wiesbaden, Germany). The output Transistor–Transistor Logic (TTL) pulses are converted with a multi-function acquisition card (PCL-836, Advantech, Taiwan). Besides, a potentiostat (Fujian Sanming HDV-7C) and LabView software are also the necessary components of our equipment. The process of blending used thermomixer comfort (Eppendorf, Hamburg, Germany). The MNPs were recovered by Pickpen (1-M, Bio-Nobile, Finland). The corresponding flow cytometer detection was carried out by BD FACSCanto II (Franklin Lakes, USA), and by FCS Express V3 analysis. Fluorescence analysis was used by Perkin–Elmer LS-55 (IN, USA). The primers for PCR amplification were designed using Primer Premier 5 software. The results analysis was performed by OriginPro 7.5.

2.3. Labeling procedures of oligo to TBR and MNPs

The TBR-DNA labeling was completed by our previous report (Zhou et al., 2009). The steady coupling of MNPs was accomplished by an optimized procedure, and could avoid the aggregation of MNPs during the labeling. MNPs solution (100 μL, 1 mg) were washed twice with 0.01 M NaOH at room temperature. The particles were then washed two times with deionized water in the same manner. The recovered MNPs were added to 90 μL MES buffer (100 mM, pH 5.0) containing 2.5 nmol 5′-amino-modified forward primer and 0.25% Tween-20, then mixed at room temperature. After 30 min, 10 μL of MES buffer including 1.91 mg of EDC was added to the MNPs solution. The solution was mixed using the rotator for another 5 h at room temperature. The primer–MNPs conjugates were washed three times with 200 μL 1× commercial PCR buffer and incubated with 0.5% BSA for 3 h at 37 °C, then recovered using a magnet Pickpen. Finally, the magnetic primer suspended in 100 μL PCR buffer containing 0.5% BSA and 0.25% Tween-20, then stored at 4 °C prior to use.

2.4. Genomic DNA extraction

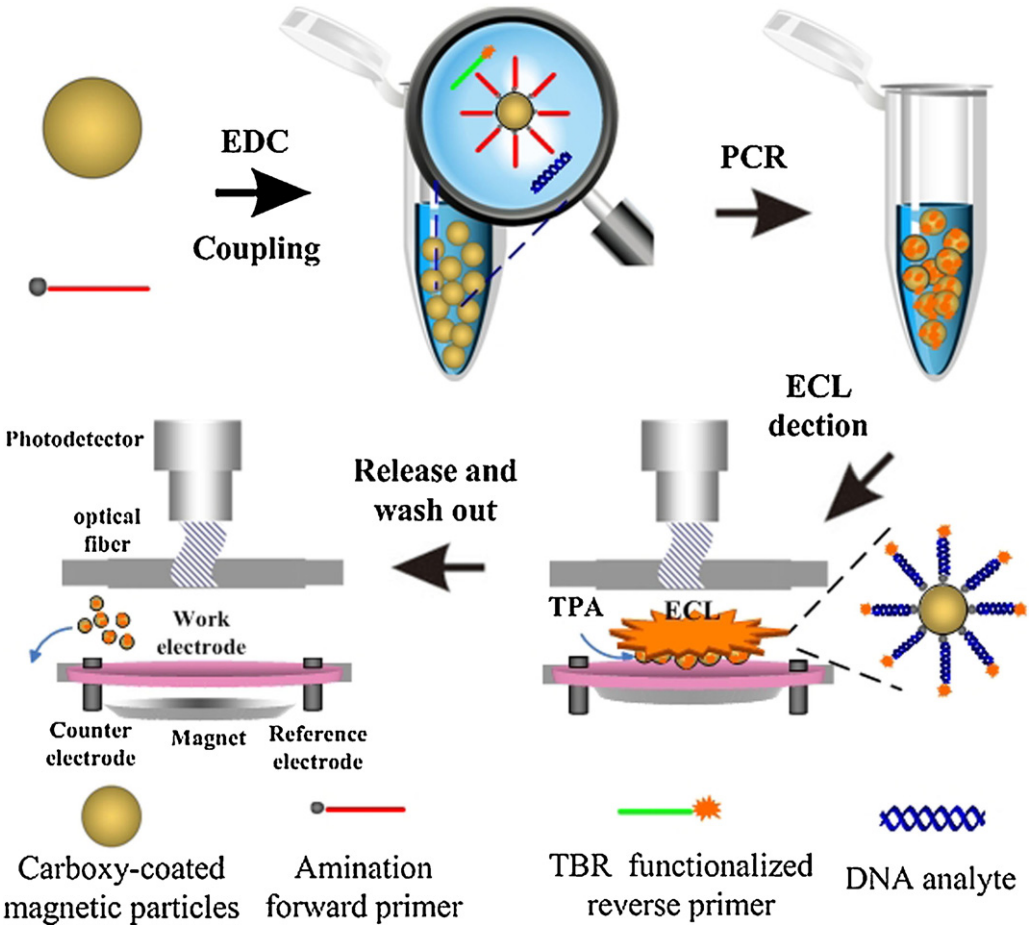
L. monocytogenes was grown in brain heart infusion broth at 37 °C with shaking overnight. The bacteria DNA isolated from 1 ml cultures were extracted according to the manufacturer's protocol from the TIANamp Bacteria DNA Kit. Then the extract was quantified by measuring the optical density at 260 nm with a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany).

2.5. The flow cytometer detection for hybridizing of MNPs and FAM probe

The MNPs were added to 100 μL PBS buffer (pH 7.4, 100 mM NaCl) containing 500 nM FAM-labeled probe 1 or 2 and 0.5% Tween-20; incubated at 92 °C for 2 min, then cooled rapidly at 50 °C; After 30 min, the MNPs were washed two times with PBS buffer, then analyzed with a 488 nm excitation source.

2.6. The protocols of PCR cycling and ECL detection

PCR was carried out in an Eppendorf AG thermal cycler with the following program: 93 °C for 2 min; 35 cycles at 93 °C for 10 s, 56 °C



Scheme 1. Schematic description of the NMPE-PCR assay.

for 10 s, and 72 °C for 10 s; 72 °C for 2 min; 4 °C hold. After amplification, the MNPs with products of PCR on its surface were collected by a magnet, and then resuspended in 100 μl ECL assay buffer (200 mM phosphate, 50 mM NaCl, 7 mM NaN_3, 0.8 μM Triton X-100, 0.4 mM Tween 20, 100 mM TPA, pH 8.0). Next, the sample was transferred to the flow ECL detection cell where the products–MNPs 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. Simultaneously, computer read the ECL signals by labview software. At last, the MNPs could be washed off immediately upon removal of the magnetic field, to achieve rapid detection of the samples.

3. Results and discussion

3.1. The principle of NMPE-PCR for detecting target gene

The basic principle of the proposed method is illustrated in Scheme 1. This assay started with the preparation of nano-magnetic primer, which employed the carboxy coated MNPs (100 nm diameter) to label with amido-labeled forward primer (Amido-F-primer) via well-known covalent coupling by EDC. In addition, TBR functionalized reverse primer (TBR-R-primer) labeling was accomplished in our laboratory. With the presence of DNA analyte and PCR mixture, the TBR labeled products were directly loaded and enriched on the surface of MNPs during thermal cycling. After magnetic separation, the MNPs–TBR complexes were resuspended in ECL assay buffer and rapidly analyzed by the custom-built ECL detection system which was described in detail in our previous research (Liu et al., 2004).

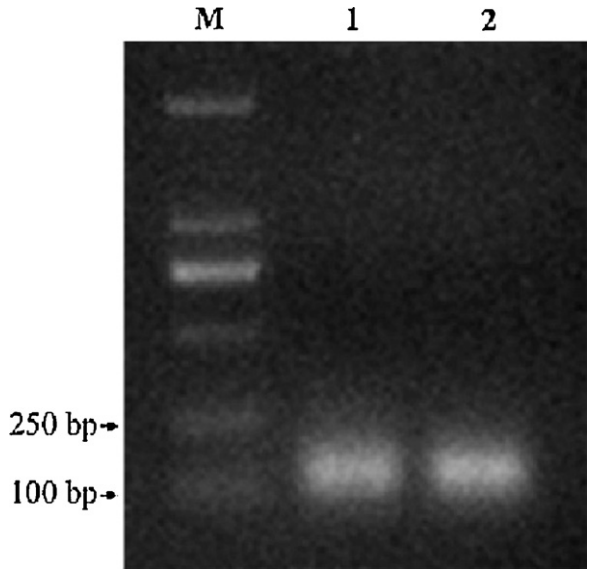


Fig. 1. Conventional 2% gel electrophoresis identified that the primers were specific in design. The DL2000 Marker was indicated in lane M. Lanes 1 and 2 represent the product of DNA segment (135 bp) which were amplified by F-primer with R-primer and TBR-R-primer, respectively. The PCR reaction was performed in 100 μL mixture containing F-primer (400 nM), R-primer or TBR-R-primer (about 400 nM), Ex Taq (2 U), commercial Ex PCR buffer, dNTP (mix 0.1 mM) and 100 ng target genome.

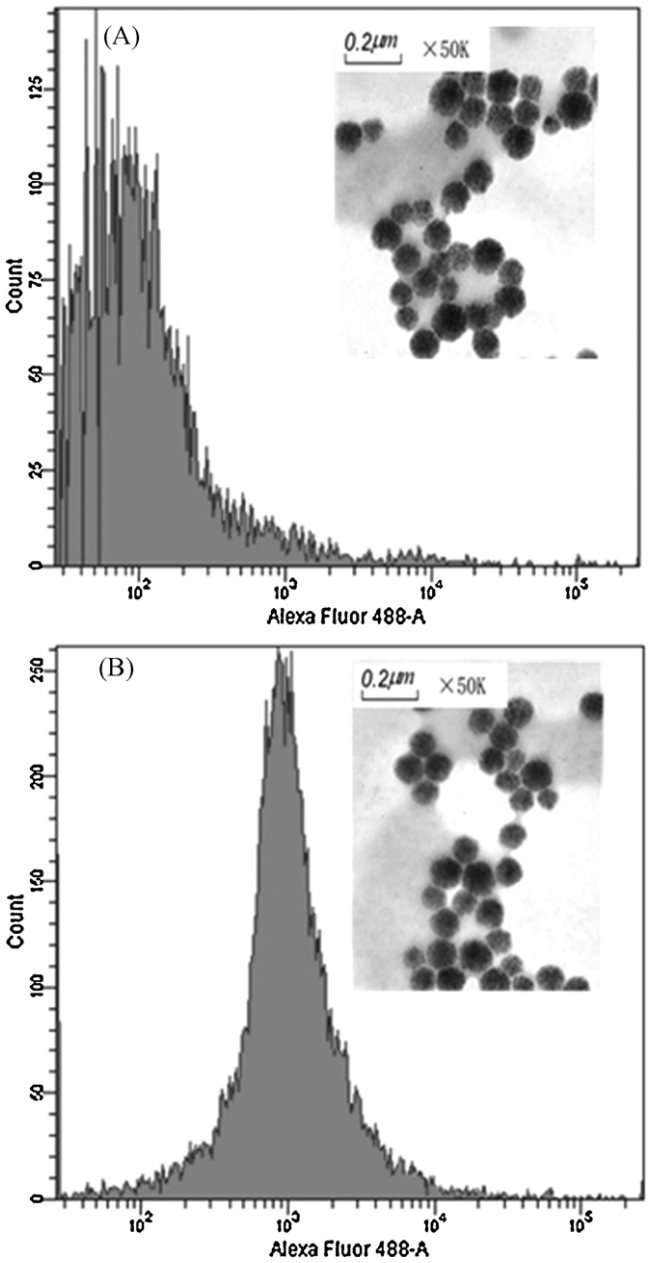


Fig. 2. The covalent link efficiency of F-primer to the MNPs is detected by annealing FAM-labeled probe 1 which is complementary to the F-primer: (A) unmodified MNPs; (B) F-primer modified MNPs. Inset: the corresponding photograph of TEM.

3.2. Design of primer for NMPE-PCR assay

We used *L. monocytogenes* as the target to examine these desirable properties of this assay. *L. monocytogenes* is one of the most frequently occurring pathogen that affects human health. The infections caused by it are a persistent and serious public problem (Mclauchlin et al., 2004; Liu, 2006; Vanegas et al., 2009). *L. monocytogenes* is estimated to be responsible for approximately 2500 cases of human illnesses, and has a 30% high fatality rate, which far exceeds other foodborne pathogens. For the application of this new approach, a couple of primers were designed on the 16S–23S intergenic region (GenBank: U57912.1, Fig. 1). In our preliminary study, we found that the nano-magnetic primer based PCR was not as efficient as the PCR with the same primer free in solution (data not shown). This is mainly because steric hindrance on the surface of particle hindered the primer and target sequence recognition.

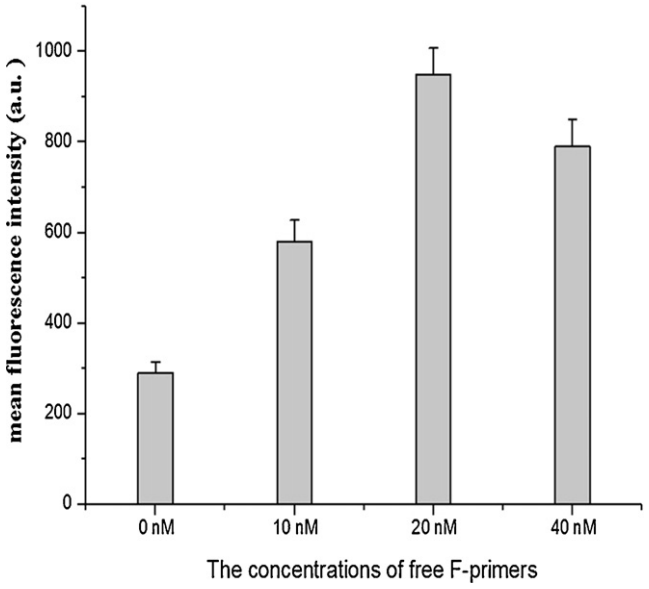


Fig. 3. Flow cytometry evaluated the average emission on each MNP after PCR with different concentrations of free F-primers: (A) 0 nM; (B) 10 nM; (C) 20 nM; and (D) 40 nM. The procedures of PCR were subjected to the following protocols: the PCR mixture contained 25 μg functionalized MNPs (blocked by BSA), 400 nM R-primer, target genome (100 ng), Ex Taq (5 U), 0.1% TritonX-100, 0.5% Tween-20, and dNTP (mix 0.1 mM).

In addition, previous reports have indicated that the efficiency of amplification on solid surface decreased with the increase of amplicon length (Diehl et al., 2006). For these reasons, we used a pair of F-primers in two forms: one was amido-labeled F-primer with inserted spacer of poly-thymine (T) linker for preparing nano-magnetic primer. Then the stability and efficiency of nano-magnetic primer have been validated by TEM and flow cytometry, respectively. Fig. 2 shows that no significant morphologic changes of MNPs occurred after coupling, while the fluorescence intensity has obvious enhancement after annealed with FAM-labeled probe 1. It indicates that the F-primer has been successfully immobilized on the surface of MNPs; the other one was non-amination F-primer, free in solution, which yielded an amplicon shorter than the original genome. This amplicon could be served as templates for further amplification cycles.

3.3. Optimization of NMPE-PCR experiment conditions

The success of the current assay is influenced by the quantity of TBR-primer and free F-primer. Excess TBR-primer included in this assay could assist the pre-amplification and maximize the probability that magnetic primer extended by polymerase on particle, and then facilitate the first few rounds of amplification of the genome (Dressman et al., 2003). As for free F-primer, if too much of it was added, fewer amplification on the MNPs would occur because of competition between magnetic primers and free F-primer in solution. Hence, the additional amount of the free F-primers should be optimized carefully. The hypothesis was evaluated by flow cytometry analysis using R-primer (instead of TBR-primer) and a FAM-labeled probe 2 which complementary to the region of PCR products. Fig. 3 has summarized that the trend change of fluorescence is related to various concentrations of free F-primers. The highest intensity of average emission on each MNP was detected when the concentrations of free F-primer was 20 nM, thus we adopted this condition in the following assay. It is also noteworthy that the tests without the genome or polymerase have obtained the similar results (Fig. S2) that the emission of MNPs is still weak after PCR and hybridization. These data suggest that the weak

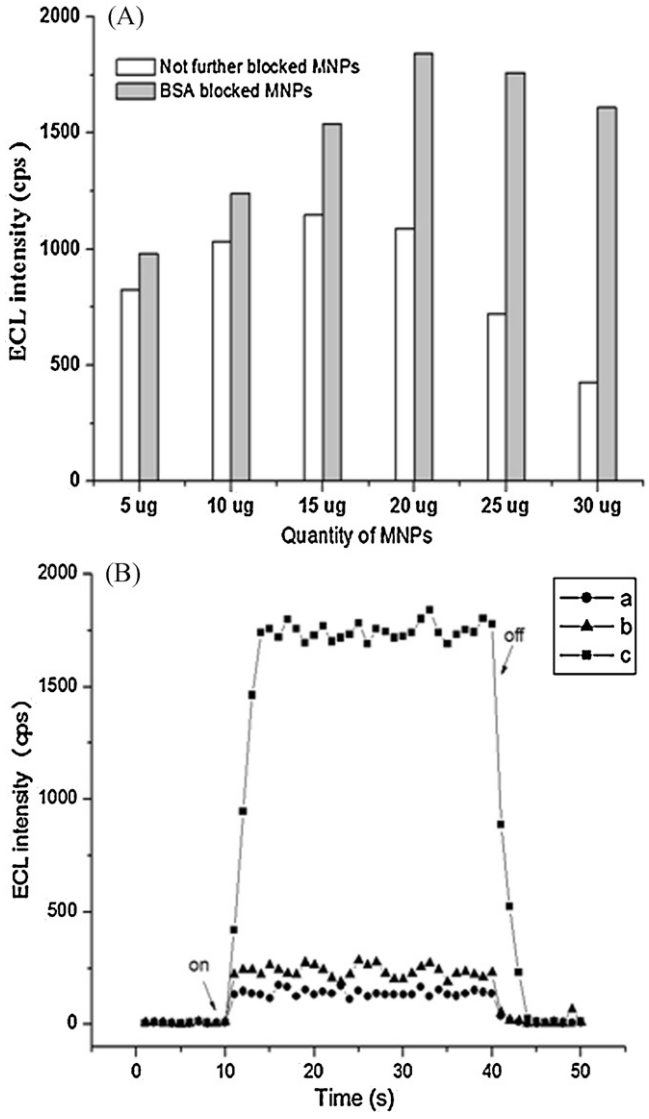


Fig. 4. (A) The effect of the quantity change and different types of primer-MNPs conjugations on NMPE-PCR assay: white bar, not blocked with BSA; gray bar: blocked with BSA. The 100 μL PCR mixture contained TBR-R-primer (400 nM), free F-primer (20 nM), target genome (100 ng), Ex Taq (5 U), 0.1% TritonX-100, 0.5% Tween-20, and dNTP (mix 0.1 mM). (B) ECL intensity corresponding to (a) ECL buffer background (TE + TPA), (b) negative control, and (c) 100 ng genomic DNA after amplification.

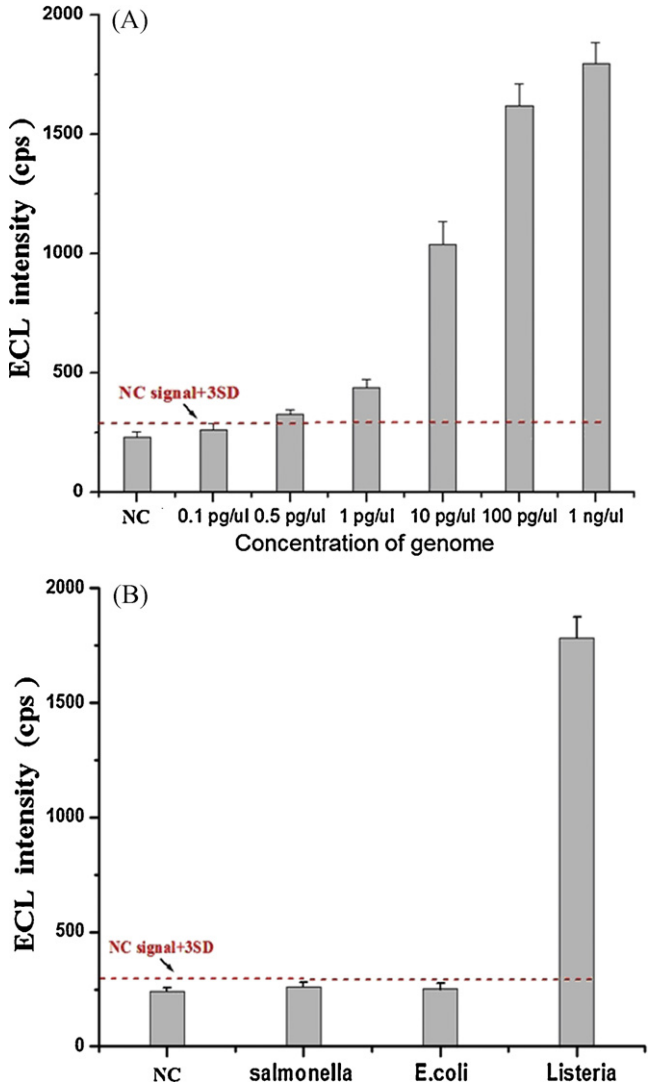


Fig. 5. (A) ECL intensity of this sensing system in the absence and the presence of different quantities of genomic DNA. (B) The variation in ECL intensity upon the addition of different genomic DNA of foodborne pathogens (100 ng).

fluorescence is not derived from nonspecific amplification but possibly due to spontaneous fluorescence of MNPs.

In order to obtain high assay sensitivity, the surface density of F-primer on the MNPs and the usage of MNPs should also be considered. We conjecture that MNPs with higher density of F-primer on its surface would provide more F-primers, thus improving the efficiency of amplification. We yet found that the higher density of nano-magnetic primer could not be stably prepared by increasing the concentration of EDC and primer in labeling process. Because MNPs would obviously aggregate in the present of high concentration of reagents, and then induced a very low recovery ratio of MNPs. Hence, we tried to obtain sufficient magnetic primers by increasing the usage of MNPs when the density of nano-magnetic primer could not be further increased at will. However, when excess MNPs were included, the fluorescence intensity of MNPs measured by flow cytometry would be weak. This phenomenon is possibly generated by superfluous MNPs adsorbing the scarce DNA and polymerase in solution. In order to validate this hypothesis and find the appropriate amount of MNPs for NMPE-PCR, we tested the ECL signal of two primer-MNPs conjugations (whether incubate with 0.5% BSA) with the quantity ranging from 5 to 30 μg in 100 μL PCR mixture. As we expect, Fig. 4A has clearly shows that the signal of experimental groups are always superior to the control groups which are not further blocked by BSA. Thus the adverse effect of adsorption on MNPs has been proved, and the maximized ECL efficiency is realized when MNPs is 20 μg.

We subsequently evaluated the qualitative results of the NMPE-PCR assay. The PCR was performed in 100 μL solution containing 20 μg functionalized MNPs, free F-primer (20 nM), TBR-R-primer (400 nM), Ex Taq (5 U), commercial Ex PCR buffer, 0.1% TritonX-100, 0.5% Tween-20, 0.05% BSA, dNTP (mix 0.1 mM) and 100 ng target genome. PCR cycling was carried out under the above-mentioned conditions. Fig. 4B shows the ECL signal obtained from analyzing TPA buffer background (a), negative control (NC) (b), sample containing 100 ng genome extracted from *L. monocytogenes* (c). As we expected, ECL values of NC is close to the value of TPA buffer background; the ECL value emitted upon analysis of the sample was 1768 counts per second (cps), which was significantly higher than the value of NC (238 cps). To further verify the in situ PCR amplification products, the same samples were also hybridized with FAM-labeled probe 2, and then detected by flow cytometry. Fig. S3

shows that the relative average emission on each particle is greatly enhanced after PCR. The corresponding result reveals that our ECL assay is credible and practicable for pathogens detection.

3.4. The sensitivity and specificity of NMPE-PCR assay

To investigate this novel method for potential application, the sensitivity and specificity of this assay were also analyzed under the optimized conditions. Fig. 5A displays the results of NMPE-PCR assays upon analysis of genome concentration range from 0 to 1 ng/μL. We note that ECL intensity increases sharply with the increase of target concentration from 1 pg/μL to 100 pg/μL. When the solution reaches a concentration of 1 ng/μL, a plateau effect is achieved. To define whether a sample is target-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 in the following formula (1):

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

According to this equation, the threshold value (NC + 3SD) was set as 299 cps. A positive sample of contamination occurrence could be discriminated easily by ECL signal higher than threshold value under our conditions, thus the limit of detection was estimated to be 500 fg/μL in an hour. As compared to previous methods for genome of pathogens, this sensitivity is higher than some time-consuming DNA-based detection platforms and isothermal replication, and could approximate to real time PCR technology (Baeumner et al., 2004; Liao and Ho, 2009; Dunbar et al., 2003). Furthermore, the specificity of this assay was also examined. We amplified the genome of some common bacterial pathogens (*E. coli O157* and *S. enterica*) with the primer pairs which were designed to target *L. monocytogenes*. Fig. 5B showed that only the *L. monocytogenes* genome (100 ng) gave a significant higher ECL value, while the ECL intensities of nonspecific analyte were similar to the NC. It indicates that the assay is responsive to its target analyte with extraordinary specificity.

4. Conclusions

In summary, we have, for the first time, demonstrated a genome-sensing strategy by integrating two in situ processes: PCR on the surface of MNPs and magnetic beads based ECL readout platform, to achieve the following virtues for analytical detection. Firstly, by taking advantage of nano-magnetic primer based in situ PCR for signal amplification, and free pretreatment of ECL for rapid detection, we have shown that the assays could be accomplished in 1 h with a high sensitivity. Compared to some conventional analytic methods for genomic samples of pathogens, such as PCR-electrophoresis, beads based ECL-PCR and isothermal replication, the proposed assay provided an time-efficient and safe approach for genome analysis. Secondly, our method requires neither complicated operations nor sophisticated assembly of instrumentation, and most of reagents and equipments are commercial. Moreover, because of avoiding some manual operation of post-modification and post-incubation, the probable error could be reduced. Thus, this low-cost and rapid assay has potential for a mass of samples detection. Lastly, our study provides the evidence as a proof-of-concept, thus opening a new direction for the development of automatic and general equipment which adopted the technology of PCR-ECL integration for performing genome analysis. We anticipate that the novel in situ NMPE-PCR assay would find numerous applications in this field.

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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.2011.11.016.

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