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Nucleic Acid Biosensor Synthesis of an All-in-One Universal Blocking Linker RPA (UBLRPA) with a PNA-based Lateral Flow Device (PLFD) for Ultra-sensitive Detection of Food Pathogens

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ABSTRACT: In this study, a whole-course nucleic-acid-constructed biosensor that combines the all-in-one concepts of the universal blocking linker recombinase polymerase amplification (UBLRPA) and a peptide nucleic acid (PNA)-based lateral flow device (PLFD) has been developed for the ultrasensitive detection of food pathogens. Using the pre-amplification UBLRPA principle, a universal linker and C3 space blocker were utilized to produce the universal linker single-duplex DNA products. The developed amplification system was employed to convert duplex products to a single strand. In the signal recognition strategy, a special PNA probe was successfully employed in the portable PLFD. The UBLRPA products were identified visually using the PLFD through dual hybridization (a PNA probe on the gold nanoparticle (Au-NP) was combined with a universal linker on the end of the products; a PNA capture probe was used on the test line, and a universal linker on the other end of the products). The accumulation of Au-NPs produced a characteristic red band, enabling the visual detection of a food pathogen without further testing. To demonstrate the value of the all-in-one biosensor, *Salmonella enterica subsp. enterica serovar typhimurium* was used as a model organism. The biosensor showed high selectivity and extraordinary repeatability using *S. typhimurium*, and the limit of detection was 4 CFU mL^{-1}. Furthermore, when milk samples artificially contaminated with *S. typhimurium* were analyzed, the analysis was completed within 30 min without complicated instrumentation. The results exhibited good precision and recovery. This portable all-in-one biosensor demonstrates great promise for the screening of pathogens in food and environmental samples.

Although bacteria are omnipresent in nature, pathogenic bacteria are potentially serious threats to health and safety. According to the World Health Organization, an estimated 2.2 million deaths annually are due to infections from food and water.[1] Rapid detection methods for foodborne pathogens have emerged to replace the conventional culture-based methods, which are time-consuming, have low-fidelity, require complex operations, and are not amenable to point-of-care testing.[2] Rapid detection technologies include enzyme-linked immunosorbent assays (ELISA),[3] qualitative and quantitative PCR,[4-6] isothermal amplification technologies,[7-9] and biosensors,[10-12]. Biosensors are highly sensitive and can rapidly and effectively detect pathogens. As an analytical tool, biosensors that transform a specific biometric event into a measurable signal are considered the best way to rapidly detect pathogens. Various biosensors have been used to detect foodborne pathogens, including optical biosensors, [13] electrochemical biosensors, [14] and piezoelectric biosensors.[15] Most of the aforementioned biosensors have good specificity and sensitivity, but their complexity and high cost make them unpopular for most end-users.[10] The advantages of nucleic acids, however, are manifold-they are lightweight, easy to synthesize, inexpensive, highly stable, versatile, and portable. The integration of nucleic acids with biosensors has been a prevailing trend in the field of sensors. Lateral flow nucleic acid biosensors (LFNABs) are an excellent candidates for screening or detecting pathogens. The construction of the LFNABs from nucleic acids may therefore be a good strategy for the detection of food pathogens.

The recognition system is an important part of LFNABs. The sandwich format of LFNABs can be classified as either antigen-antibody or nucleic acid hybridization. With the advantages of high specificity and selectivity, the use of antibody-based LFNABs is a common strategy to detect double-stranded amplicons, which are dual-labeled and captured by a primer with two different tags. However, the labels are complicated, expensive, intrusive, and easily interfered with during amplification.[16] Furthermore, in the field of portable pathogen detection, the limited stability of antibodies is the greatest challenge to the extensive application of the labeled biosensors.[17] Nucleic acid hybridization-based LFNABs are an excellent alternative for analyzing single-stranded amplicons with an immobilized complementary probe. The two types of oligonucleotide probes are detector probes and capture probes, which must simultaneously hybridize to complementary sequences within the same target.[18] Moreover, different kinds of probes are available, the most common of which is deoxyribonucleic acid (DNA).[19] Peptide nucleic acid (PNA), a special nucleic acid with a peptide-like backbone, can also be utilized in nucleic acid hybridization.[20] The bases of PNA are connected by methylene carbonyl linkages, but PNA still contains six

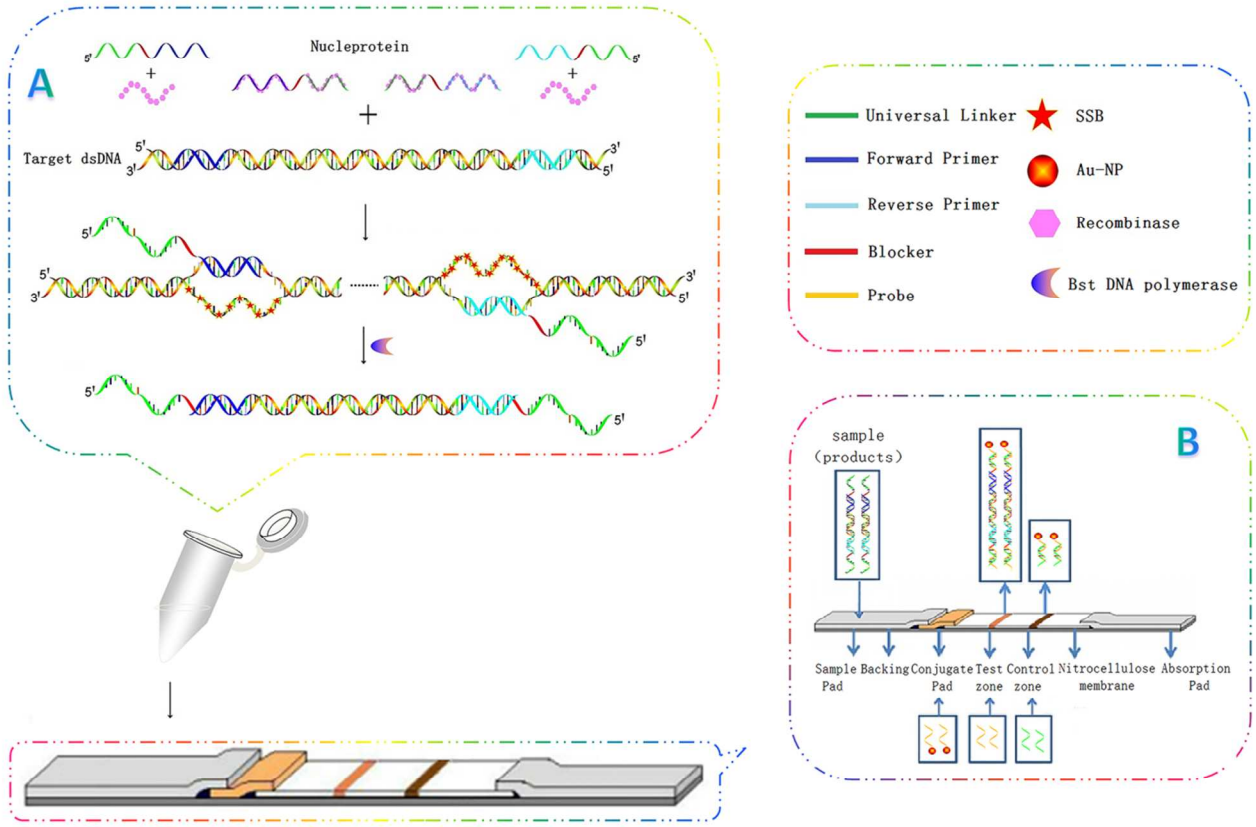
backbone bonds between bases and three bonds between the bases and backbones. Such a structure provides both PNA and PNA/DNA with high biological stability, even in harsh conditions. [21] Obviously, a strong captor is critical to transducing and amplifying the measurable signal in a sandwich bioassay. Since they have neutral backbones, PNA probes have greater stability and affinity than DNA, and PNA/DNA hybridization will enable LFNABs to achieve higher detection sensitivity.

A pre-amplification step can be used to obtain more product than would otherwise be available. Generally, the obtained signal includes an original proportion of the target in direct detection without amplification. In contrast, the amplification step may provide an opportunity to scale up the terminal signal and thus is an effective approach for improving sensitivity. To further improve the sensitivity and specificity of LFNABs, a pre-amplification step is commonly adopted using classical PCR or isothermal amplification, and LFNAB is then used to detect the amplified product. Considering the timeliness required for point-of-care testing (POCT), recombinase polymerase amplification (RPA) is the preferred technique for simple and fast nucleic-acid-based diagnosis. Additionally, the reaction can be performed in a single lyophilized pellet in a considerably shorter time (typically <15 min).[22] This method is based on two specific oligonucleotide primers and a mixture of recombinase, polymerase, and single-stranded DNA binding proteins (SSBs), which can bind to the template and generate double-stranded products. The primer can also be designed with a specific cleavage site and labeled with a fluorophore and quencher to enable monitoring the reaction in real time. RPA has been described in several reports on the amplification of DNA from bacteria and viruses.[23] To adapt the double-stranded products of RPA to the LFNABs, a labeled reverse primer and a lateral flow probe were used to generate dual-antigen–labeled products that can be captured by the LFNABs.[24] This would mean that the analysis of double-stranded amplicons is limited by the less stable antibody-based LFNABs. Asymmetric PCR is rou-tinely used after symmetric PCR for ssDNA amplification but has low sensitivity.[25] Therefore, new amplification strategies that can generate a single-stranded product from a double-stranded input have become a main research focus to expand the range of highly sensitive LFNABs applications, especially label-free applications.

Recently, abasic sites were used in label-free biosensors to overcome the limitations of labeled biosensors.[26] A tetrahydrofuranyl residue (dSpacer), which lacks a nucleobase moiety, was originally developed to design an abasic site.[27] Alternatively, a C3 spacer, which can prevent nucleobases from polymerizing during amplification, can also be utilized.[28, 29] This bioinspired strategy of selectively terminating amplification provides a blueprint for an improved pre-amplification step, which can yield a special product that has partially-complementary strands and a linked single strand. In addition, the special products can be captured and fixed onto the LFNABs through hybridization between the captured probe and the single-stranded nucleic acid. Coincidentally, we previously reported [30] that a novel universal primer originally developed as a linker can be converted into a universal platform for designing label-free DNA sensors. We hypothesized that a new type of pre-amplification method could prepared by producing a specific product that can be recognized by a nucleic acid-based LFNAB.

To investigate this hypothesis, we designed an all-in-one nucleic acid biosensor (Scheme 1) that included a universal blocking linker-RPA (UBLRPA) signal amplification approach and a PNA-based lateral flow device (PLFD) for the rapid and supersensitive detection of *Salmonella*. In this study, the newly designed nucleic acid biosensor can convert biomass to a nucleic-acid-detectable signal with functional nucleic-acid-controlled signal identification, amplification and output. Importantly, this universal and ultrasensitive biosensor has great potential for detecting other food pathogens, as it can provide useful information for research into risk assessment and the regulation of food safety.

Scheme 1 Design Strategy of the all-in-one nucleic acid biosensor system. (A) The pre-amplification strategy for the Universal Blocking Linker RPA (UBLRPA); (B) The signal output principle of the PNA-based lateral flow device (PLFD).



EXPERIMENTAL SECTION

Reagents and materials. A TwistDx TwistAmp Basic kit was purchased from TwistDX Ltd. (TwistAmp Basic, TwistDx, Cambridge, UK). A deoxynucleotide (dNTP) mix was ordered from New England Biolabs (Ipswich, MA, USA). The following reagents were obtained from Sigma (St. Louis, MO, USA): chloroauric acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$), trisodium citrate, sodium citrate dehydrate, sodium dodecyl sulfonate (SDS), NaCl, yeast extract, tryptone, bismuth agar (BS), $Na_3PO_4 \cdot 12H_2O$, Tween-20, sucrose, phosphate-buffered saline (PBS, pH 7.4, 0.01 M), mycose bovine serum albumin (BSA), sodium chloride–sodium citrate (SSC) buffer (20 concentrate, pH 7.0), Triton X-100, and borate buffer (BB, pH 9.0, 0.1 M). Streptavidin was purchased from Invitrogen (Carlsbad, CA, USA). Doublye distilled water (ddwater) was used in all experiments.

All LFNAB materials were purchased from Millipore (Bedford, MA, USA), including backing cards (HF000MC100), glass fiber sample pads (CFSP001700), conjugation pads (GFCP000800), nitrocellulose membranes (135 s) and absorbent pads (CFSP001700).

Samples and preparation. *Salmonella* spp. and other bacterial strains were supplied by the China Center of Industrial Culture Collection (CICC), the National Center for Medical Culture Collection (CMCC), the American Type Culture Collection (ATCC), and the Laboratory of Food Safety at the College of Food Science and Nutritional Engineering at China Agricultural University. *Salmonella enterica subsp. enterica serovar typhimurium* (*S. typhimurium*, CICC 10420) was used as a model organism to demonstrate the efficacy of the all-in-one biosensor analysis. All strains were stored in 20% (v/v) glycerol solution at −80 °C until they were used. Food products were purchased at a local supermarket.

All strains were grown in Luria-Bertani broth (LB: 5 g NaCl, 5 g yeast extract, 10 g tryptone, and H_2O to 1,000 mL) medium at 37 °C for 16 h. The *S. typhimurium* inoculation assay was prepared by adding 10-fold serial dilutions of each pathogen in 0.8% NaCl, ranging from 1 to 10^6 CFU mL^{-1}. Genomic DNA templates were extracted with a Bacteria DNA Kit (Tiangen, Beijing, China).

Primer design. RPA primers to targete the invasion protein gene *invA* (GenBank: DQ644633.1) of *Salmonella* spp. were designed according to the Appendix of the TwistAmp™ reaction kit manuals.[31] Five primer sets were used in the experiment. In the UBLRPA strategy, the universal linker (UL) and C3 space blocker were attached to the 5' end of the RPA primers. To obtain a UL single-duplex DNA product, the number of the blocker was set to 0, 1, 2 or 4, and the red letter "X" highlight was introduced in the primer sequence to mimic an abasic site. In addition, all probes, including the control line capture probe (CCP), test line capture probe (TCP) and ligation-probe (P) of Au-NPs that were used in PLFD, were all synthesized in PNA. To ensure that the availability of PLFD, the control line should always be visualized as a characteristic red band during analysis such that the sequence of CCP on the control line is consistent with the UL. Notebly, the N-terminus of CCP was modified with biotin (BIO) for immobilization to the control line. The N-terminus of TCP was also modified with BIO to prevent movement on the test line. In addition, P had the same sequence as TCP, but a cysteine residue (Cys) was added to the N-terminus of P to provide the thiol necessary to immobilize the Au-NPs. A continuous glutamic acid tail was added to the C-terminus. All DNA and PNA sequences used are shown in Table S1 and were synthesized by KareBay Biochem, Inc. (Ningbo, China).

DNA amplification by RPA and Universal Blocking Linker -RPA (UBLRPA). To demonstrate the effectiveness of RPA primers and optimize the system for RPA, the basic infor-

mation for the reactions was improved. RPA amplification was carried out with the TwistAmp Basic kit (TwistDX, Cambridge, UK) in a Bio-Rad S1000TM thermal cycler (BIO-RAD, USA). Each reaction was performed in a 47.5-μL reaction mixture containing 29.5 μL of rehydration buffer, 2.4 μL of forward and reverse primers (10 μM each) and 2 μL of the DNA template. Then, the reaction mix was added to the freeze-dried tube, and the tube was flicked until the freeze-dried powder fully dissolved. To initiate the reaction, 2.5 μL of 280 mM magnesium acetate (MgAc) was added. Since the RPA reaction started as soon as MgAc was added, the tubes were immediately incubated for 25 min (typically) at 38 °C in a thermal cycler. UBLRPA use the same reaction system as RPA. The amplified products were analyzed using 2% agarose gel electrophoresis. Three biological replicates were conducted, and each biological replicate was analyzed.

Synthesis of Au-NPs, compound Au-NPs and ligation-probe. Citrate-stabilized Au-NPs with an average diameter of ~13 nm were prepared using the previously described method with $HAuCl_4$ and trisodium citrate.[32] Briefly, Au-NPs were prepared in 50 mL of aqueous solution. Then, 1.67 mL of 1% trisodium citrate was added to a boiling 2.4×10^{-4} M $HAuCl_4$ solution, stirred for 30 min cooled to room temperature and filtered through a 0.45-micron nylon filter (Osmonics). The diameter of the Au-NPs was characterized with TEM images, the plasmon absorption band was exhibited by UV–vis spectrum, and the zeta potential of the Au-NPs was determined at pH=7 with the ZetaPlus Zeta Potential Analyzer from Brookhaven Instruments Corporation.

Au-NPs modified by PNA ligation-probes were prepared using a modified method according to previous literature.[33, 34] To inhibit coagulation, Au-NPs were stabilized by complexation with *p*-bis-sulfonatophenyl (phenylphosphine) (BSPP). Then, 5 μM PNA probes (P) with 10 nM Au-NP suspensions were incubated in 5 mM phosphate buffer for 12 h. After incubation, the PNA-modified nanoparticles (Au-NPs-P complex) were centrifuged at 14,000 rpm for 20 min, and the supernatant was discarded. Finally, the Au-NPs-P complexes were washed twice with 5 mM phosphate buffer and dispersed in 10 mM phosphate buffer (containing 10 nM NaCl). After centrifugation of the Au-NPs-P complex, the monodispersity, plasmon absorption and zeta potential of the Au-NPs were observed using TEM imaging, UV–vis spectrum and the ZetaPlus Zeta Potential Analyzer, respectively.

Preparation of PNA-based lateral flow device (PLFD). The PLFD was assembled with four parts on a plastic backing, including the sample pad, conjugate pad, NC membranes and absorption pad from bottom to top. The sample and conjugate pads were made from glass fiber, saturated with a buffer (containing 0.25% Triton X-100, 0.1 M BB, and 0.15 M NaCl, pH 8.0) and dried at 37 °C for 2 h. The streptavidin and biotinylated PNA were dissolved in solubilizing buffer (PBS containing 0.5% mycose). Streptavidin-biotinylated TCP/CCP conjugates were obtained by mixing 30 μL of 1 mg/mL of streptavidin solution with 30 μL of 100 μM PNA probe solution, which was incubated at room temperature for 1 h. Then, 30 μL of each conjugate was affixed to the nitrocellulose membrane using a BioDot BioJet BJQ 3000 dispenser (Irvine, CA), generating the test line and control line. The NC membrane was dried at 37 °C for 12 h after being modified with PNA and then stored at 4 °C.

Detection of *Salmonella* spp. with the all-in-one biosensor. For the all-in-one biosensor strategy based on UBLRPA and PLFD, 2.5 μL of the Au-NPs-P complexes was loaded onto the conjugate pad, and a 50-μL running buffer containing 4×SSC, 2% BSA and 0.05% Tween-20 (pH 7.0) with 10 μL of the UBLRPA product solution was applied to the sample pad. During the assay process, the solution with the Au-NPs-P complexes migrated through the test line and control line in sequence due to capillary force. Subsequently, the presence of the UL single-duplex DNA products in the solution was visually determined within 5 min. The intensities of the red bands on the test strip were quantified with a portable strip reader.

For the recovery study, ultra-high temperature (UHT) milk (purchased from Carrefour supermarket) was applied. To ensure that the milk was totally sterile, *S. typhimurium* was first cultured and counted. *S. typhimurium* was spiked into the milk at the concentrations of 5, 10^1, 10^3 and 10^5 CFU mL^{-1}. For this biosensor strategy, 4 contaminated milks were analyzed, and DNA was extracted from the spiked samples with the Bacteria DNA Kit (Tiangen, Beijing, China). The milk samples spiked with *S. typhimurium* were also measured with the standard culture method. The spiked milk (2.5 mL) was added to 22.5 mL sterile sodium citrate buffer (10 mM, pH 7.0) and shaken for 1 min. Then, 1 mL of mixture was transferred to the BS and incubated at 36 ± 1 °C for 40-48 h.

RESULTS AND DISCUSSION

Design of UBLRPA-PLFD Sensing System. In this report, we describe the construction of an all-in-one biosensor that combines the amplification features of UBLRPA with visual detection on a PLFD. The strategy for UBLRPA (Scheme 1A) is used to generate numerous single linker-attached duplex DNAs. The primer includes a nucleic acid segment (specific primer, dark/light blue) complementary to the *invA* gene of *Salmonella* and is linked using a C3 spacer blocker (abasic site, red) and a universal nucleic acid sequence. Recombinase proteins assemble along the oligonucleotide primers to form a stable helical filament (protein-DNA complex). This complex is highly invasive to homologous sequences and forms a D-loop structure at the cognate site, which exposes the 3'-end of the primer. The unwinding DNA is stabilized by SSBs to prevent the ejection of the primer by branch migration. Subsequent to polymerization, continuous strand displacement amplification occurs in the presence of the polymerase, and the polymerization is always terminated at the abasic site. As a result, free single-stranded nucleic acid residues are generated to yield the complementary DNA of the PNA-containing probes at both ends of the duplex products. Subsequently, the generated single-duplex DNA complexes were visually analyzed on the PLFD, as shown in Scheme 1B. Via linkage to streptavidin, the biotin-CCP was directly sprayed onto the nitrocellulose membrane of the control line. Another biotin-TCP was also immobilized on the test line via the biotin-streptavidin linkage. The ligation of Cys-P and Au-NPs led to the formation of the Au-NPs-P complex loaded onto the conjugate pad. While the solution was applied on the sample pad, the complex migrated forward with the solution due to capillary action. In the presence of positive products, hybridization among the immobilized TCP, the UL single-double strand products, and the Au-NPs-P complex all appear on the test line. The accumulation of Au-NPs was visualized on the test line as a red band. The excess Au-NPs-P complexes continued to migrate and were captured by the immobilized CCP on the control line, forming a second red band. Typically, in a sample solution without positive products, no Au-NPs-P complexes accumulated on the test line. Such results were indicated by the presence of only one red band on the control line.

Screening of a candidate RPA primer. RPA primers are typically 30 to 35 nucleotides long; however, primers of to 45 nucleotides have been successfully used in the RPA process. Cytidines at the first 3-5 nucleotide positions of the 5' end may help facilitate the formation of recombinase filaments. Additionally, guanines and cytidines, which tend to improve per-

formance, may provide a more stable 'clamped' target for the polymerase at the last 3 nucleotide positions of the 3' end. Avoiding long tracks of one particular nucleotide or a large number of small repeats is recommended.[31] In accordance with these guidelines, five sets of primers were designed, and all were applied in the experiment under different reaction conditions. After the reaction mix was added to the freeze-dried tube, it was subjected to three types of treatment: first, pipetting to mix and then adding the MgAc (refer to the TwistAmp Basic Quick Guide); second, pipetting to mix, incubating at 38 °C for 4 min and then adding the MgAc (refer to the TwistAmp Basic Quick Guide 'For low template copy number'); and third, flicking the tube to fully dissolve freeze-dried powder until transparent and then adding the MgAc (this study). The results of RPA are shown in Fig. S1; the first treatment had no product (Fig. S1A), and the second treatment had positive products with extensive tailing (Fig. S1B). The third treatment exhibited clearly positive bands, especially primer 4 (Fig. S1C). We concluded that RPA-F4/R4 was ideal for modification with a UL and blocker to detect *Salmonella spp.* in the UBLRPA assay.

Establishment of the UBLRPA. The UL was a G-rich DNA sequence, particularly at the first 3-5 nucleotide positions at the 5' end, which might inhibit the recombinase. To assure the influence of amplification efficiency with the UL, the genomic DNA of *S. typhimurium* was used to assess the feasibility using four sets of primers: RPA-F4 and RPA-R4, RPA-UF0 and RPA-R4, RPA-F4 and RPA-UR0, and RPA-UF0 and RPA-UR0. Each band was clearly observed using 2% agarose gel electrophoresis (Fig. 1A), indicating that each target fragment was amplified effectively. The length differences between the adjacent targets were easily detectable at 19 bp and 38 bp, which agreed with the length of UL. Subsequent results showed that the G-rich UL would not prevent the synthesis of protein-DNA complexes. This may be because the specific primer was highly effective at combining with recombinase. Under these circumstances, the specific primer could also invade the homologous sequences, even if the UL did not form a protein-DNA complex. To obtain a UL single-duplex DNA product with the complex primer RPA-UF0 and RPA-UR0, an abasic site was designed between the UL and the specific primer, with a C3 space blocker. The number of blockers was set as 1, 2 and 4. The products were analyzed using 2% agarose gel electrophoresis (Fig. 1B) and an Agilent 2100 Bioanalyzer (Agilent Technologies) (Fig. S2). However, the blocker and no-blocker primers were indistinguishable in the agarose gel electrophoresis. This was likely due to the low resolution of the agarose gel which could not effectively distinguish the single-double strand complex. Hence, the Agilent 2100 Bioanalyzer was chosen to help parse the complexities of each product. As shown in Fig. S2, prospective products were observed in an UBLRPA assay utilizing a complex primer with blockers, whereas no blocker was observed for the other treatment. To ensure fidelity, RPA-UF2/UR2 was used in subsequent experiments. Genomic DNA from the 16 strains was used to determine the specificity of the UBLRPA assays (Fig. 1C). Positive reactions were observed for all *Salmonella* spp., whereas no positive results were observed for other strains.

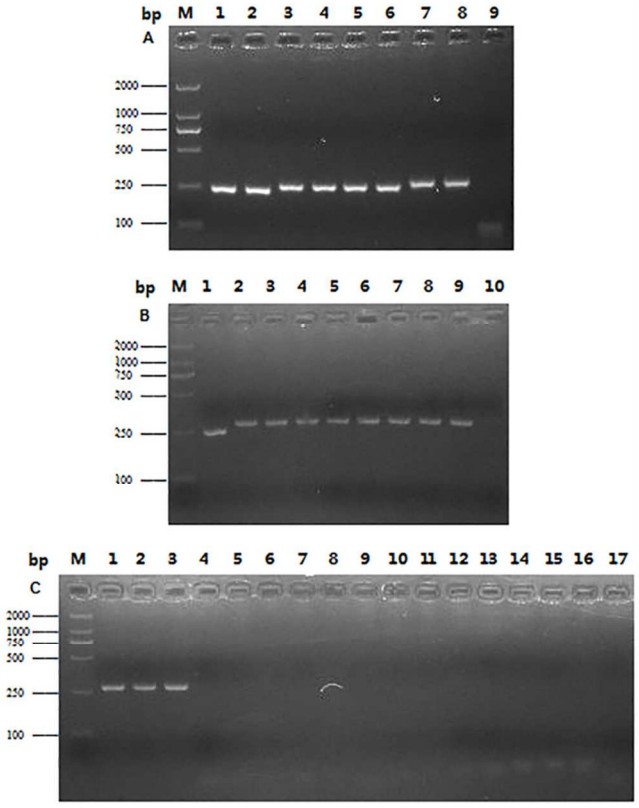


Figure 1. The electrophoretograms for the UBLRPA strategy. (A) The feasibility of compound primers with two parallel. Lane M: DNA Marker DL 2000. Lane 1-2, primer RPA-F4 and RPA-R4; 3-4, primer RPA-UF0 and RPA-R4; 5-6, primer RPA-F4 and RPA-UR0; 7-8, primer RPA-UF0 and RPA- UR0; 9, negative control. (B) The validation of an abasic site in a primer with two parallel. Lane M: DNA Marker DL 2000. Lane 1, positive control; 2-3, no C3 space blocker; 4-5, one C3 space blocker; 6-7, two C3 space blockers; 8-9, four C3 space blockers; 10, negative control. (C) The specificity of UBLRPA assays. Lane M: DNA Marker DL 2000. Lane 1, *Salmonella typhimurium* CICC 10420; 2, *Salmonella enterica* ATCC 55105; 3, *Salmonella spp.* CGMCC 1.1552; 4, *Listeria monocytogenes* ATCC 19112; 5, *Listeria monocytogenes* CAU 05021; 6, *Pseudomonas aeruginosa* ATCC 47085; 7, Escherichia coli CICC 10899; 8, *Staphyloccocus aureus* CICC 10306; 9, *Clostridium perfringens* ATCC 10388; 10, *Bacillus cereus* ATCC 21928; 11, *Enterobacter sakazakii* ATCC 51329; 12, *Shigella sonnei* CICC 21535; 13, *Bifidobacterium longum* CGMCC 2265; 14, *Acetobacter aceti* ATCC 15973; 15, *Zygosaccharomyces bailii* ATCC 56075; 16, *Bacteroides fragilis* ATCC 25285; 17, negative control.

Characterization of Au-NPs and Au-NPs-P. Transmission electron microscopy (TEM) was used to examine the size and morphology of the obtained Au-NPs and Au-NPs-P, and the results are shown in Fig S2A and Fig S2B, respectively. As can be seen, Fig 2C shows the UV−vis absorption spectra recorded from AuNPs and AuNPs modified with PNA. As for the independent AuNPs, the UV–vis spectrum exhibited a characteristic plasmon absorption band with a maximum at 520 nm. However, in the case of Au-NPs modified by the PNA probes after centrifugation, from which the surface plasmon absorption of Au-NPs-P slightly shifted to 525 nm were observed. As shown in Fig. S3A, the average diameter of the Au-NPs was approximately 13 nm, an optimal amount for ligating PNA.[34] Furthermore, the TEM image of Au-NPs-P (Fig. S3B) showed a fuzzy

edge, indicating that many PNAs surrounded the Au-NPs. As shown in Fig. 2, the initial zeta potential values of the AuNPs and PNA ligation-probes at pH 7 were -35.1 mV and -7.32 mV, respectively. After conjugation, PNA ligation-probes covered the entire surface of the AuNPs, and the zeta potential of the Au-NPs-P complex became -15.2 mV at pH 7, which was close to the zeta potential of the pure PNA ligation-probes. The measured zeta potentials demonstrated the successful conjugation of PNA ligation-probes to AuNPs.

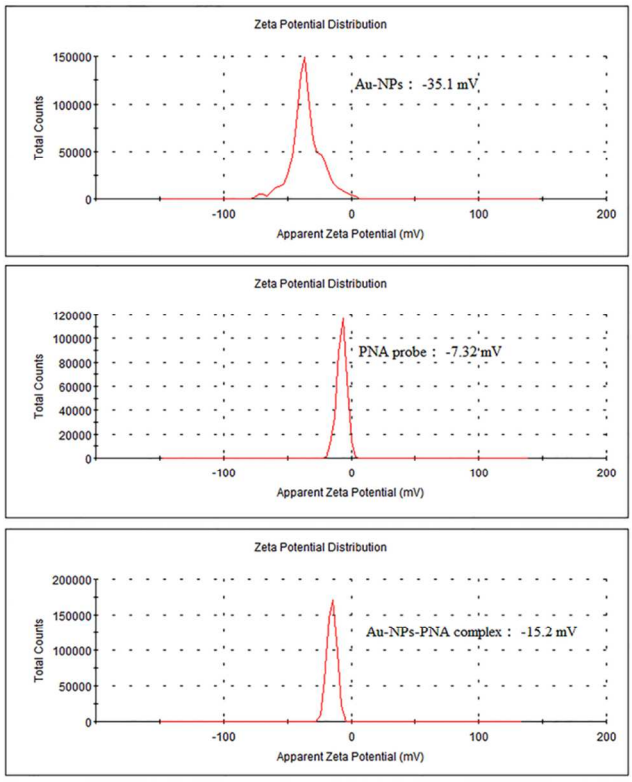


Figure 2. Zeta potential of AuNPs, PNA ligation-probes and Au-NPs-P complex.

Combinations of PNA and DNA. Isothermal titration calorimetry (ITC) is a technique for measuring the thermodynamics of two solutions. It is a powerful tool for analyzing the kinematics of complexation in biological systems. ITC is widely used in protein-protein, protein-DNA, and protein-lipid interaction studies.[35] The PNA strand has a high sequence preference to hybridize with complementary ssDNA, RNA, or another PNA, forming highly stable duplexes with a high degree of sequence selectivity. In this study, ITC was used to directly record the energetics of the interaction of PNA with DNA and the corresponding interaction with DNA. To determine the best capture probe for the products in question, the length of the probe was tested at 16, 14 and 12 nt. Running buffer solution (70 μM) containing probes, and a DNA solution (15μM), were placed in the titration syringe and the sample cell, respectively. Aliquots of the probe solution (2.5 μL) were added (a total of 20 injections) to the sample solution 300 s apart. As seen in Fig. S4 and Table S3, the probe with 14 nt PNA showed optimal performance. As shown in Fig. 3A, a binding event between the PNA and DNA was observed with the apparent equilibrium constant K_d = 5.599 E-5 M, and a PNA/DNA stoichiometry of 1.212. Results from the ITC experiment curve shown in Fig. 3B show that DNA bound to DNA with a lower apparent equilibrium constant K_d = 3.845 E-4 M, with a DNA/DNA stoichiometry of 1.096. Therefore, a strong bridging interaction between the PNA and DNA existed, consistent with the reported results.[36] The hybrid thermal stabilities decreased for identical sequences in the order PNA−PNA > PNA−RNA > PNA−DNA > DNA−DNA.

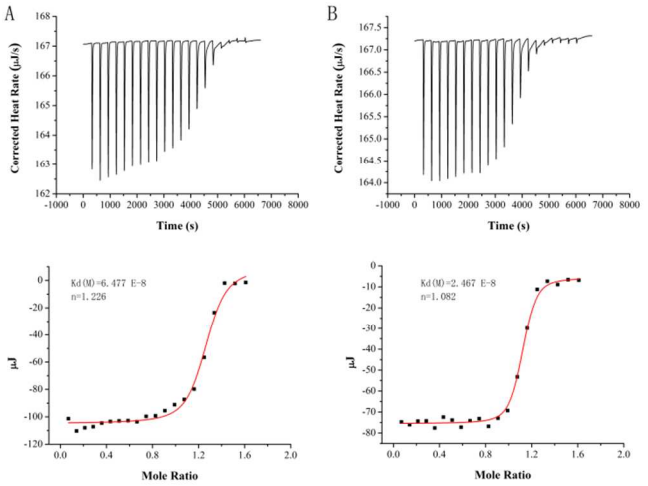


Figure 3 ITC data curves and binding isotherms for titration of DNA with (A) PNA and (B) DNA.

PLFD assay performance. To evaluate the effectiveness of the experiment, reviewing the analytical performance (specificity, selectivity, reproducibility and stability) of PLFD in biological samples was essential. To study the specificity of the PLFD, a series of genomic DNA preparations, including *Salmonella* spp. and other bacterial species, were tested after UBLRPA. As shown in Fig. S5, only *Salmonella* spp. showed any detectable color intensity on the test line. These results demonstrated an excellent selectivity of the proposed strategy for *Salmonella* spp. analysis against other relevant potential bacteria, which suggests that the developed PLFD has good selectivity and specificity for *Salmonella* spp. and can be readily applied for detection in real biological samples.

Under the UBLRPA conditions, the sensitivity of the PLFD was tested with 10-fold serial dilutions of *S. typhimurium* samples (ranging from 10^0 to 10^6 CFU mL^{-1}). Images of PLFD in response to various concentrations are shown in Fig. 4A. The color intensity of the test line increased with the increasing concentration of *S. typhimurium*; the sensitivity of the quantitative test was 10 CFU mL^{-1}. Subsequently, the limit of detection (LOD) was tested with serial dilutions of *S. typhimurium* samples (10、8、6、4、2 CFU mL^{-1}), and the LOD was determined to be 4 CFU mL^{-1}(Fig. 4B). For quantitative measurements, the PLFD was inserted into the portable "strip reader" instrument DT2050 purchased from Shanghai Goldbio Tech. Co., Ltd. (Shanghai, China), and the optical intensities of the red lines were recorded with "Goldbio strip reader" software, which could determine parameters, such as peak height and area integral. Each sample was detected 3 times, and the average value of three measurements was used. (Data was shown in Table S4). The resulting data was applied for the quantitative analysis of *S. typhimurium*. As shown in Fig. 4C, the resulting plot of the response to *S. typhimurium* concentration was linear from 10 to 10^6 CFU mL^{-1}, and the correlation equation was Peak Area = 2727.86 lg $_{S.\ typhimurium}$ + 20032.86 (correlation coefficient: R^2 = 0.99046). For visual detection, the red band in the PLFD test line was observed with a concentration as low as 4 CFU mL^{-1}.

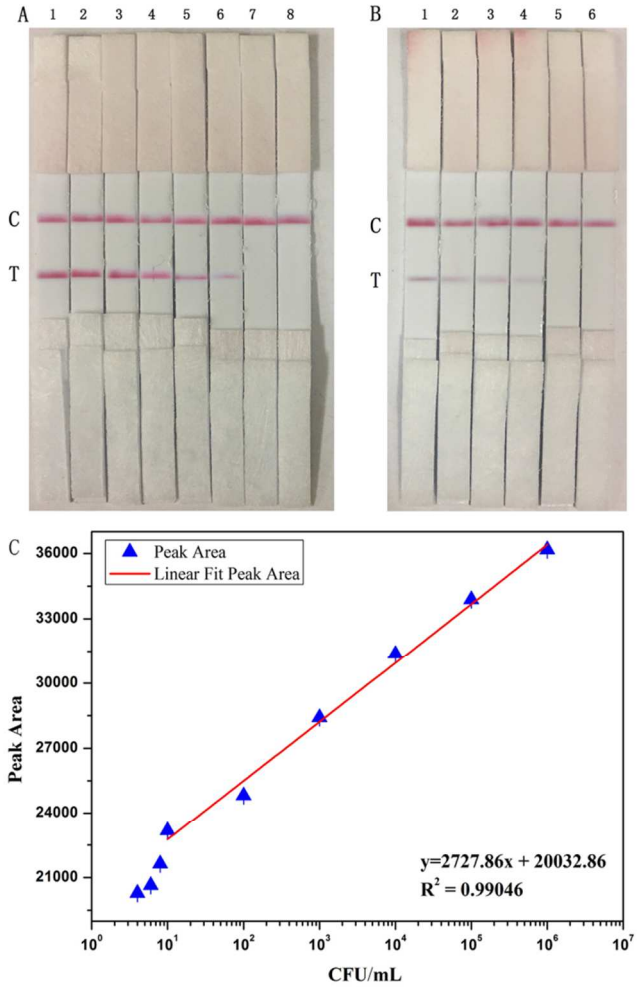


Figure 4. Analysis of the UBLRPA products with the PLFD. (A) Sensitivity test. Lane 1, 10^6 CFU mL-1; 2, 10^5 CFU mL-1; 3, 10^4 CFU mL-1; 4, 10^3 CFU mL^{-1}; 5, 10^2 CFU mL^{-1}; 6, 10 CFU mL^{-1}; 7, 1 CFU mL^{-1}; 8, negative control. All pictures were collected after 3 min. (B) Limit of detection. Lane 1, 10 CFU mL^{-1}; 2, 8 CFU mL^{-1}; 3, 6 CFU mL^{-1}; 4, 4 CFU mL^{-1}; 5, 2 CFU mL^{-1}; 6, negative control. All pictures were captured after 3 min. (C) The scatter diagram between the red color intensity and different concentrations of S. typhimurium. The points of dynamic range are presented by a triangle shape. The standard curve was inserted to illustrate the liner relationship.

Reproducibility is one of the most important criteria in assessing the PLFD system. The reproducibility of the proposed PLFD was evaluated by testing sample solutions in the presence of *S. typhimurium* (10^2, 10^4 and 10^6 CFU mL^{-1}). As shown in Table S5, similar responses were obtained for each concentration. The corresponding RSD values of the optical responses for 10^2, 10^4 and 10^6 CFU mL^{-1} were 0.06%, 0.03% and 0.04%, respectively, indicating the excellent analytical reproducibility of the measurements. For the stability study, the performance of the PLFD was intermittently measured (every 2 weeks) over three months of storage in Ziploc bags at room temperature. As shown in Fig. S6, the responses of PLFD on the test bands performed over 3 months were still valid. The intensity of the test bands for detecting *S. typhimurium* (10^2 CFU mL^{-1}) was almost the same as that obtained with the newly made system, indicating that the PLFD had good stability.

Application of the all-in-one biosensor for detecting *S. typhimurium* in milk. To further test the feasibility in practical use, only the biosensor was applied to analyze *S. typhimurium* in real milk samples. For the recovery and practical sample studies, *S. typhimurium* was detected in artificially contaminated milk samples at concentrations of 10^1, 10^3 and 10^5 CFU mL^{-1}. In one test, DNA was extracted from 3 contaminated milks and was analyzed by the proposed biosensor. For the other test, the samples were homogenized in 22.5 mL of sodium citrate buffer and then analyzed using the standard culture and colony counting method (data not shown). Recoveries of *S. typhimurium* were in the range of 118.5 ± 0.9% to 135.4 ± 4.3% (Table 1). For the real sample qualitative study, milk was artificially contaminated with 5 CFU mL^{-1} *S. typhimurium*, and these levels were also detected. Using this method, determining whether the milk samples were contaminated with *Salmonella* was easy. The results indicated that the all-in-one biosensor could be a great resource for testing in the field.

The biosensor only targets DNA amplification and determination, not DNA extraction. However, many rapid genome extraction methods, such as the boiling method and magnetic isolation method, among many others, are available. Reischl et al. developed a boiling procedure for total DNA extraction from *S. aureus* by thermal lysis. [37] This boiling method offers many benefits, including rapid, simple, and effective analysis, especially for gram-negative bacteria. This method has been widely used in many previous studies. [38-40] However, the DNA quality was generally low, which would decrease the LOD in this study. A novel genomic DNA isolation method developed by Alghuthaymi et al. involving magnetic nanoparticles (MNPs) probes provided reasonably high adsorption of DNA. [41] The magnetic isolation method has advantages over commercial kits and the boiling method, including its high quality, simple treatment, quick processing, and its chemical-free, environmentally-friendly process. However, the price of MNPs will increase the total cost of detection. This rapid method does not require special instrument and can be finished in a few minutes. When bacteria levels are low, it is effective in extracting trace amounts of the genome. Therefore, the conformity of the DNA rapid extraction method represents a direction for future development.

Table 1. Recoveries of S. typhimurium-spiked milk.

Original value ($CFU \cdot ml^{-1}$)	*S. typhimurium* added ($CFU \cdot ml^{-1}$)	*S. typhimurium* tested ($CFU \cdot ml^{-1}$)	Recovery (%)
0	1×10^1	$(1.354 \pm 0.043) \times 10^1$	135.4 ± 4.3
0	1×10^3	$(1.182 \pm 0.007) \times 10^3$	118.2 ± 0.7
0	1×10^5	$(1.185 \pm 0.009) \times 10^5$	118.5± 0.9

CONCLUSION

In summary, an innovative whole-course nucleic-acid-constructed biosensor has been developed to rapidly detect *Salmonella* app., integrating the all-in-one pre-amplification of ULBRPA with the signal recognition of PLFD. All reactions were performed in a sealed system regardless of the amplification process or products recognized. Employing the UL and C3 space blocker, a ULBRPA technology-based assay was devel-

oped with excellent sensitivity and simplicity. The most important contribution of this UL is to achieve the all-in-one transition, in which the products obtained from the various bacteria all had the same single-strand UL. At the same time, the innovative complex (UL and C3 space blocker) ingeniously solved the problem of converting dsDNA into ssDNA. As expected, a UL single-duplex DNA product was successfully obtained in the ULBRPA strategy and could be examined using PLFD. The PLFD platform used a PNA probe to hybridize the UL, forming a more stable PNA/DNA compound. It then accumulated gold nanoparticles by capillary action, providing a colorimetric signal. The developed all-in-one biosensor was shown to respond linearly over the *S. typhimurium* concentration range of 10 to 10^6 CFU mL^{-1}, with an LOD of 4 CFU mL^{-1}. Due to the neoteric ULBRPA, the biosensor can be widely used to rapidly detect food pathogens. Due to the strong binding between PNA and DNA, the biosensor displays high sensitivity and specificity. Due to the PLFD, the biosensor becomes more portable, thus being amenable to on-site detection in future. Therefore, the combination of the ULBRPA and PLFD provides excellent convenience for the fast detection of *Salmonella*. In addition, the developed approach can also be applied in for detecting other food pathogens using appropriate primers. In brief, as a portable and sealed biosensor for detecting food pathogens, this biosensor has excellent prospects for the on-site safety assessment of environmental and food products.

ASSOCIATED CONTENT

Supporting Information

Table S1-S5 and Figures S1-S6, as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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All authors have given approval to the final version of the manuscript.

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