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Polyethersulfone-Based Microfluidic Device Integrated with DNA Extraction on Paper and Recombinase Polymerase Amplification for the Detection of *Salmonella enterica*

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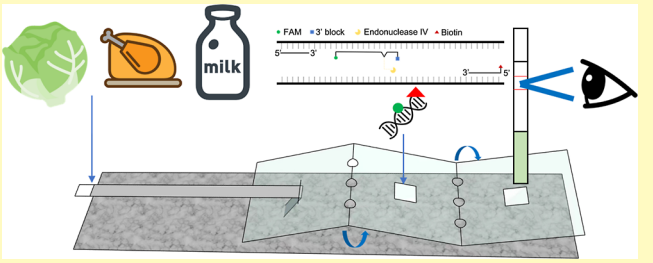
ABSTRACT: Rising consumption, large-scale production, and widespread distribution have been accompanied by an increase in the number of *Salmonella* infections reported to implicate contaminated food products. We developed a portable origami microfluidic device that enabled rapid detection of *S. enterica* from sample preparation to end-point detection, including nucleic acid extraction on paper dipstick without pipetting, nucleic acid amplification using isothermal recombinase polymerase amplification (RPA), and lateral flow assay for results readout. We also explored the feasibility of the polyethersulfone (PES) membrane as a new reaction matrix against the widely used chromatography paper to optimize nucleic acid amplification. Nucleic acid amplification was achieved within 20 min and demonstrated 100% specificity to *S. enterica*. The limit of detection of this PES-based microfluidic device was 260 CFU/mL and equivalent to RPA reaction in tube. A chromatography paper-based microfluidic device was found 1-log less in sensitivity for *Salmonella* detection compared to the use of PES. This PES-based microfluidic device could detect *S. enterica* in lettuce, chicken breast, and milk at concentrations of 6 CFU/g, 9 CFU/g, and 58 CFU/mL, respectively, after 6 h enrichment. PES has shown high compatibility to isothermal nucleic acid amplification and great potential to be implemented as an integrated sample-to-answer microfluidic device for the detection of pathogens in various food commodities.



KEYWORDS: *microfluidic "lab-on-a-chip", recombinase polymerase amplification, DNA extraction, food safety, pathogen, Salmonella*

Foodborne diseases have posed significant health and economic burdens to the public, and the impact is comparable to other illnesses, such as HIV/AIDS, malaria, and tuberculosis.[1] Rising consumption, large-scale production, and widespread distribution have been accompanied by an increase in the number of foodborne infections reported to implicate contaminated food products. In 2015, the World Health Organization (WHO) reported *Salmonella enterica* as the first rank responsible for foodborne mortalities among 22 bacterial (e.g., enterotoxigenic *Escherichia coli*, *Campylobacter* spp., etc.), protozoal (*Cryptosporidium* spp., *Giardia lamblia*, etc.), and viral agents (norovirus, hepatitis A virus, etc.).[2] The transmission route of *Salmonella* to humans is associated with diverse food products, such as poultry, eggs, vegetables, and milk.[3,4] In response to increased *Salmonella* infections, rapid and accurate detection methods are required for both surveillance purpose and outbreak investigation on site. Traditional culture-based methods cannot satisfy the requirement of point-of-need analysis due to the lengthy recovery cycle of bacteria and complex detection procedure.[5,6] The ideal point-of-care (POC) device has been characterized by WHO as affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free, and deliverable (ASSURED).[7] These criteria are in line with the concept of microfluidic "lab-on-a-chip", which can manipulate small volume of fluids within the channels of small dimensions on a customized platform.[8]

The choice of substrate material is vital to the characteristics of microfluidic "lab-on-a-chip" devices and has evolved from silicon and glass to polymers, such as polydimethylsiloxane (PDMS) and methyl methacrylate due to ease of fabrication, reduced cost, and rapid prototyping.[9,10] After a prominent invention of paper-based microfluidics from the Whitesides Group at Harvard University, cellulose paper has been validated as appropriate in disease diagnostics and environmental monitoring.[11,12] Paper constitutes interwoven cellulose fibers and forms three-dimensional porous structure for easy immobilization of molecules.[13] In addition, fluids can be wicked through paper substrate via capillary force, which eliminates the need of an external pump to drive.[14] Paper is ubiquitously available, extremely low cost, and sustainable.

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Table 1. RPA Primers and Probe Sequences Targeting the *invA* Gene of *Salmonella enterica*

primer name	nucleotide sequences (5′−3′)	amplicon size (bp)
probe S2	FAM-CGAATTACGAGCAGTAATGGTATCTGCTGAAGTTGAG-THF-ATGTTATTCGC-C3 spacer	
reverse R21	Biotin-GATCTTTATGTGCAATCAATAAATCATCCAAC	
forward F22	GAAAAAGATGTCATTAACCTTGTGGAGCATA	247
forward F23	AAGAGAAAAAGATGTCATTAACCTTGTGGAG	251
forward F24	AGAGAAAAAGATGTCATTAACCTTGTGGAG	250
reverse R25	Biotin-TTCAATCATTTTCTTAATAAATCGACGGACAT	
forward F26	TAATGGTATCTGCTGAAGTTGAGGATGTTAT	206
forward F27	ATGGTATCTGCTGAAGTTGAGGATGTTAT	204

However, several studies reported the disadvantages of paper matrix in hindering the diffusion of liquids and decreasing reaction kinetics, especially when multiple layers of paper were overlaid.[15,16] Linnes and co-workers compared the performance of chromatography paper, polycarbonate, glass microfiber, nitrocellulose, and polyethersulfone (PES) for *in situ* nucleic acid amplification.[17] Among them, PES demonstrated the best compatibility with isothermal nucleic acid amplification.

Nucleic acid-based detection approaches prevail in detecting microbes, in particular polymerase chain reaction (PCR), which traces the genomic information of targeted bacteria and offers high specificity and sensitivity.[18] However, the bulky and energy-consuming PCR machine to enable repeated heating cycles limits its application in POC diagnostics and against the demand for sustainability. Recombinase polymerase amplification (RPA) is an isothermal nucleic acid amplification method to detect DNA and RNA without the need for a separate step to produce cDNA.[19] It has the features of simple primer design and operation, fast reaction time, high sensitivity, and no need of initial high temperature to denature duplex template nucleic acids.[20,21] RPA applies two key proteins, namely, recombinase protein from *E. coli* bacteriophage T4 and single-strand DNA binding protein (SSB) to replace complex temperature regulation.[22] Depending on the initial template copy number and amplicon size, RPA reaction is usually carried out at a single temperature normally at 37−42 °C for 5−20 min. Among all isothermal amplification methods [e.g., loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), etc.], RPA can be carried out in the fastest manner and requires the lowest amplification temperature.[23] Relatively low reaction temperature and short amplification time strengthen the potential applications of RPA in the POC diagnostics. To achieve a simple visualization of nucleic acid amplicons in the POC device, commercial lateral flow assay has been coupled with isothermal amplification due to its outstanding simplicity, sensitivity, and specificity. By labeling the primers with tags (e.g., 5′ FAM antigen and a 3′ biotin), nucleic acid amplicons could be captured by streptavidin at the detection line of the lateral flow strip, and generate distinct color for visualization.[15]

In the current study, we first evaluated the performance of RPA on the PES membrane. Then, we explored the potential of fabricating the PES membrane into a hybrid miniaturized foldable microfluidic chip, where simplified sample preparation and DNA extraction were integrated. *S. enterica* was used as the targeted bacterial model to validate the detection performance of this microfluidic device. The overall detection time was within 30 min to allow for rapid detection of pathogens on site and/or in the field.

MATERIALS AND METHODS

Bacterial Strains and Preparation of Genomic DNA. A total of 14 bacterial strains were used in this study. Except for *Campylobacter jejuni*, all other bacterial strains were recovered and cultivated on Luria Bertani (LB) agar (BD, Franklin Lakes, NJ, USA) at 37 °C under aerobic conditions. *C. jejuni* was cultivated on Mueller−Hinton agar supplemented with 5% defibrinated sheep blood (MHBA) (Burlington, MA, United States) at 42 °C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Overnight bacterial culture was prepared and adjusted to a pre-determined optical density OD_{600} = 0.3 (~5×10^8 CFU/mL). The bacterial count was enumerated using the conventional plating assay. Genomic DNA of bacteria for specificity and sensitivity test was prepared by thermal treatment. In brief, bacterial culture was boiled at 100 °C for 10 min and DNA was directly extracted from the lysed solution without additional centrifugation. Genomic DNA samples were stored at −20 °C for further use.

DESIGN OF RPA PRIMER AND PROBE

RPA primers and probe were designed on the conserved sequence of the *invA* gene (GenBank Accession No: MK017941 and NC003197) specific to *S. enterica*. Based on the design manual guideline of TwistAmp nfo assay (Coldhams Ln, Cambridge, UK) and in consideration of amplicon size, primer length, and GC contents, a probe and two reverse primer-derived groups of primer candidates were selected (Table 1). The specificity of the designed primers was assessed by performing primer BLAST in the NCBI database. The sequences of all primer and probe candidates were synthesized by Sangon Biotech (Shanghai, China).

RPA reaction was conducted using a TwistAmp nfo kit with some modifications on the formula. Rehydration buffer (29.5 μL) and DNase-free water (8.2 μL) were added into a dry pellet in the kit that contained lyophilized RPA reaction components (e.g., recombinase proteins, crowding reagents, etc.). After thorough mixing, 18.1 μL of the reagent mixture was transferred to a 0.2 mL tube containing 1.05 μL of forward primer (10 μM), 1.05 μL of reverse primer (10 μM), 1.05 μL of probe (1.25 μM), and 2.5 μL of DNA template. Magnesium acetate (1.25 μL) was then added to initiate the reaction. The tube was quickly vortexed and spun before incubation at 40.6 °C for 20 min in an oven (Thermo Fisher Scientific, BC, Canada). The products were examined by 3% agarose gel electrophoresis stained with SYBR Safe DNA gel stain (Thermo Fisher Scientific, USA). The gel was imaged using the Bio-Rad ChemiDoc MP imaging system (Mississauga, Ontario, Canada). Lateral flow dipstick (LFD) (Milenia HybriDetect) was purchased from Milenia Biotec (Germany) and applied to detect RPA products in the field. One microliter of the amplicons was diluted into 99 μL of Tris-buffered saline at room temperature. LFD was immersed into the liquid mixture and run for 5 min to show the color on both the test

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Table 2. Bacterial Strains Used for Specificity Tests and the Results of Lateral Flow Assays by Two Primer Pair Candidates

		RPA electrophoresis		LFA	
species	strain	R21-F23	R25-F26	R21-F23	R25-F26
Salmonella enterica	Typhimurium SL1344	+	+	+	+
Salmonella enterica	Typhimurium LT2	+	+	+	+
Salmonella enterica	Enteritidis PT4	+	+	+	+
Salmonella enterica	Enteritidis PT30	+	+	+	+
Salmonella enterica	Enteritidis ATCC43353	+	+	+	+
Salmonella enterica	Typhi TY2	+	+	+	+
Salmonella enterica	Typhi TY21a	+	+	+	+
Arcobacter butzleri	CCUG30485				
Arcobacter cryaerophilus	AF1899				
Campylobacter jejuni	ATCC33560				
Escherichia coli	O157:H7 ATTC43890				
Helicobacter pylori	ATCC43504				
Listeria monocytogenes	ATCC191113				
Staphylococcus aureus	Newman				

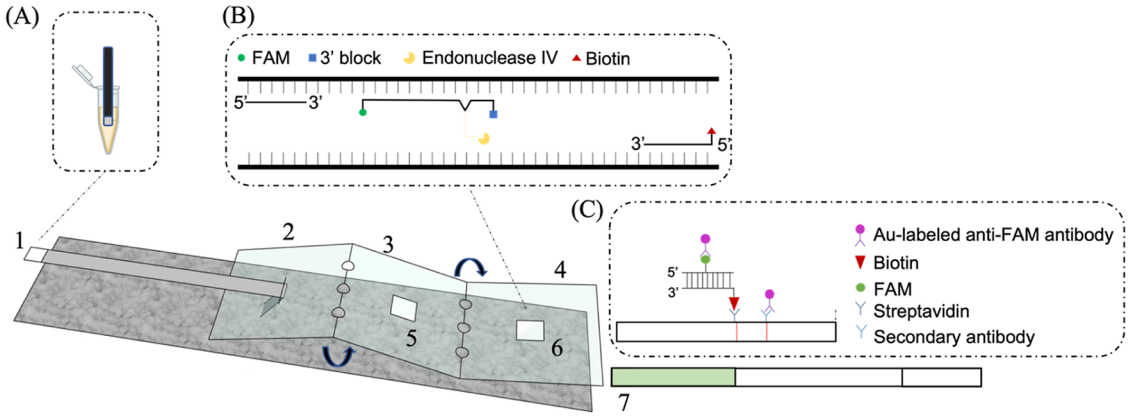

Figure 1. Schematic illustration of the PES-based microfluidic "lab-on-a-chip" device. Layers 2, 3, and 4 are made of optical adhesive film. Layer 1 contains a cellulose paper dipstick 1 with wax-printed at handle and DNA absorption at the non-waxed area. PES membrane 5 adheres at the center of layer 3 to load magnesium acetate. PES membrane 6 adheres on layer 4 symmetric to PES membrane 5 to load primers, probe, and reaction reagents. The booklet-like punched edges between layers 2, 3, and 4 are designed for folding convenience. (A) DNA extraction on cellulose paper dipstick. Paper dipstick pokes through a cut on layer 2 for flexible extension. (B) RPA reaction on PES membranes. After PES membranes 5 and 6 stack together with cellulose paper dipstick 1 inserted in between, the RPA reaction initiates. The final format of the device from top to bottom: layer 2 (folded reversely at the back of layer 3), layer 3 (faced downward), cellulose paper dipstick 1, layer 4 (faced upward). (C) Amplicon detection on LFD. Running buffer is added on PES membrane 6 and wicks through LFD 7 for result visualization.

line and control line, indicating a positive result. Otherwise, a negative result is acquired if only the control line displayed color.

Evaluation of the RPA Assay for the Detection of *S. enterica*. The specificity of the RPA assay was evaluated using DNA extracted from 7 *S. enterica* strains and 7 non-*Salmonella* strains (10^6 CFU/mL), as described in Table 2. The sensitivity of the RPA assay was evaluated using DNA extracted from *S.* Typhimurium SL1344 ranging from 2.6×10^5 to 2 CFU/mL. Sterile water was added to replace *Salmonella* DNA as the negative control (NTC). Amplicon products were analyzed by both 3% agarose gel electrophoresis and LFD. Both specificity and sensitivity tests were repeated at least in triplicate.

Design and Operation of a PES-Based Microfluidic "Lab-on-a-Chip" Device. The schematic illustration of the PES-based microfluidic device is shown in Figure 1, and the representative image is shown in Figure S1. This microfluidic device contains a cellulose paper dipstick, two PES membranes, and three layers of adhesive films. The pattern of the cellulose paper dipstick was designed by AutoCAD 2016 (Autodesk Inc., USA) and made by Whatman no. 1 filter paper. The cellulose paper dipstick was 3×53 mm^2 with one end (3×3 mm^2) painted without wax and the handle part painted with wax using a Xerox Phaser 8560N wax printer (Xerox Canada, Toronto, ON). Layers 2, 3, and 4 were squares (20×20 mm^2) made by MicroAmp optical adhesive films (Life Technologies). A 3 mm cut was at the center of the non-sticky side of layer 2 for cellulose paper dipstick to poke through and stretch flexibly. PES membranes (20×20 mm^2) 5 and 6 were placed in the middle of the sticky sides of layers 3 and 4, respectively. The edge between layers 2 and 3 and layers 3 and 4 were cut into three holes by a hole punch (Bostitch office HP12) to reduce mechanical resistance between films when folding and stacking together.

In brief, RPA reagents were pre-mixed and added to the PES membrane 6, whereas magnesium acetate was added on the PES membrane 5. The unwaxed part of the cellulose paper dipstick was dipped into a crude DNA mixture for 1 min and folded onto the PES membrane 5. Meanwhile, layer 2 was reversely folded to the back of layer 3. Layer 3 was then folded onto layer 4 and two PES membranes overlapped each other with cellulose paper dipstick inserted in between. Finally, three

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adhesive films stacked on top of each other, and the reaction was initiated at 40.6 °C for 20 min. After incubation, 100 μL of the elution buffer was added onto the PES membrane 6 on layer 4. A sample pad of LFD was then dipped in the liquid, allowing it to wick through the LFD for final visualization.

The LFD test was imaged by an iPhone 10 (Apple), and the test line intensity was measured by ImageJ (2.1.0/1.53c) as a ratio of the actual test line band intensity to the control line band intensity. ANOVA tests were conducted to determine the significant difference among the samples with concentrations from high to low and the NTC.

Performance Validation of a Microfluidic Device by Foods Contaminated with *Salmonella*. *Lettuce.* Romaine lettuce was purchased from local grocery stores. Decontamination of lettuce samples was conducted by 75% (v/v) ethanol on the surface for 15 min. After three washing cycles with deionized water to remove ethanol residue, lettuce was dried in a biosafety cabinet for 45 min at 22 °C. *S.* Typhimurium was cultivated overnight and adjusted to OD_{600} = 0.3, followed by a series of 10-fold dilutions. Each lettuce sample (25 g) was inoculated with 100 μL of *S.* Typhimurium with concentrations of 1.6×10^2 to 1.6×10^5 CFU/mL and air-dried in a biosafety cabinet for 15 min, followed by rinsing with 225 mL of phosphate-buffered saline (pH ∼ 7.4) in a sterile plastic bag prior to filtering through Whatman no. 1 paper discs (diameter 2 cm) to remove excessive rinse solution. Filter discs were enriched in a test tube containing 5 mL of LB broth for 6 h at 37 °C. The number of *S.* Typhimurium was determined by the plating assay. One milliliter of bacterial suspension was used for DNA extraction, followed by detection using the PES-based microfluidic "lab-on-a-chip" device. An NTC was included to confirm no contamination of the lettuce sample. The tests were performed at least in triplicate.

Chicken Breast. Raw and boneless chicken breast meats were purchased from local grocery stores and stored at −20 °C until use. Thawed chicken breasts were cut into pieces (25 g) and decontaminated by 1% (w/v) chlorine (NaClO) for 15 min. After 3 washing cycles with 75% (v/v) ethanol and 3 washing cycles with sterilized water, chicken breasts were dried in a biosafety cabinet for 45 min at 22 °C. *S.* Typhimurium was cultivated overnight and adjusted to OD_{600} = 0.3, followed by a series of 10-fold dilutions. The surface of each chicken breast sample was inoculated with 100 μL of *S.* Typhimurium with concentrations of 2.2×10^2 to 2.2×10^5 CFU/mL and air-dried in a biosafety cabinet for 15 min, followed by rinsing with 10 mL of phosphate-buffered saline (pH ∼ 7.4) in a sterile plastic bag prior to filtering through Whatman no. 1 paper discs (diameter 2 cm) to remove excessive rinse solution. Filter discs were enriched in a test tube containing 5 mL of LB broth for 6 h at 37 °C. The number of *S.* Typhimurium was determined by the plating assay. One milliliter of bacterial suspension was used for DNA extraction, followed by detection using the PES-based microfluidic "lab-on-a-chip" device. An NTC was included to confirm no contamination of the chicken breast sample. The tests were performed at least in triplicate.

Milk. Pasteurized partly skimmed milk (2%) was purchased from local grocery stores and stored at 4 °C. *S.* Typhimurium culture was prepared in a range of 58 to 5.8×10^4 CFU/mL, and 300 μL of each bacterial sample was separately inoculated into 3 mL of milk. The number of *S.* Typhimurium was determined by the plating assay. After incubation at 37 °C for 6 h, 1 mL of milk sample was used for DNA extraction, followed by detection using the PES-based microfluidic "lab-on-a-chip" device. An NTC was included to confirm no contamination of the chicken breast sample. The tests were performed at least in triplicate.

RESULTS AND DISCUSSION

Optimization of RPA Reaction. A probe and two reverse primers with associated forward primers were designed and are listed in Table 1. The performance of each pair was compared and optimized with different ratios of forward/reverse primer to probe (i.e., the concentration ratio of forward and reverse primers, primer: probe ratio of 1:0.25 and 1:0.125), as shown in Figure 2. All primer combinations produced strong bands, in

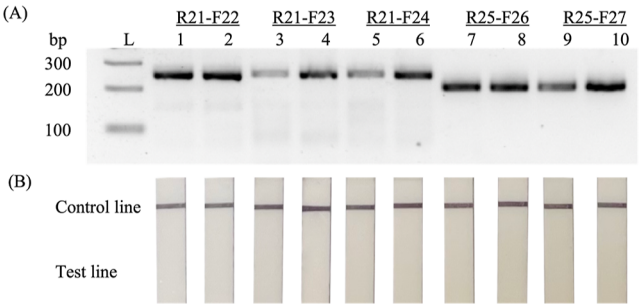

Figure 2. Selection of primer candidates with different primer: probe ratios. (A) Agarose gel image of RPA reaction with *Salmonella* DNA template. (B) LFD results of RPA reaction without the DNA template. The top line of the dipstick is the control line, and the bottom line is the test line. Samples from left to right: Lanes 1−2 are primer set R21-F22; Lanes 3−4 are primer set R21-F23; Lanes 5−6 are primer set R21-F24; Lanes 7−8 are primer set R25-F26; Lanes 9−10 are primer set R25-F27. Primer: probe ratios for each primer pair are 1:0.25 and 1:0.125 from left to right. Both reverse primer-dependent groups (R21-F22, F23, F24, and R25-F26, F27) generated stronger positive signals and clean negative results on LFD.

which R21-F23, R21-F24, and R25-F27 showed more amplification when probe concentration was 1/8 of the forward primer or reverse primer. In comparison, R21-F22 and R25-F26 showed similar amplification results from two different probe concentrations. Dual gel bands were observed and occasionally occurred when only probe attached to the complementary sequence and served as a third primer for replacement to the forward primer. After cleavage by endonuclease IV at the abasic site, the elongation started at the free 3′-OH end of the probe, resulting in incomplete amplicons.[22] For example, primer pair R21-F22 (Lanes 1 and 2) produced both complete targeted amplicon (247 bp) and a short R21-probe amplicon (167 bp) on the agarose gel. Whether primer artifacts were generated or not was assessed with the absence of *Salmonella* DNA in the RPA assay and detected on LFD. None of the primer sets generated positive results on LFD (Figure 2B), eliminating the possibility of false-positive readouts caused by primer noise.

Till date, there is no well-designed protocol or automated software for RPA primer design. Current primer design software for PCR, such as *Primer3*,[24] is not appropriate for RPA primer development, as RPA primer is longer in sequence length; needless to mention is the specific requirement of the probe.[25] The RPA kit (i.e., TwistAmp) manufacturer only provides general suggestion about primer design.[25] Specifically, (1) guanines (G) and cytidines (C) content of the primers should be between 30 and 70%, and long track of G at the 5′

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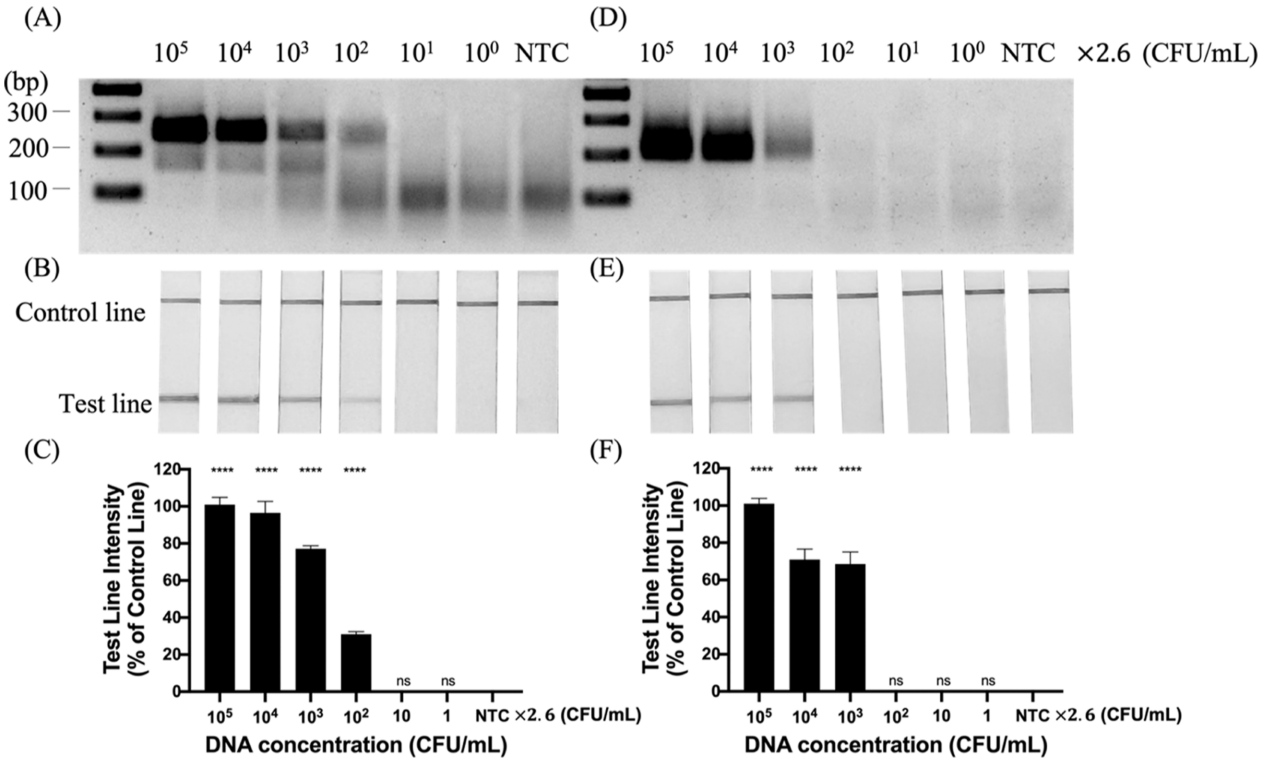

Figure 3. Sensitivity tests of the RPA assay in tube [A−C): R21-F23; (D−F): R25-F26] with final DNA concentration from 2−2.6 × 10^5 CFU/mL. (A) LOD of R21-F23 pair was 260 cfu/mL; (D) LOD of R25-F26 pair was 2600 CFU/mL. (B,E) Sensitivity of LFD for R21-F23 and R25-F26, respectively. The top line of the dipstick is the control line, and the bottom line is the test line. The LOD of LFD displayed identical to the agarose gel. (C,F) LFD test line intensity as the percentage of control line intensity (%) and statistically compared to the NTC (****$P < 0.0001$, ns = not significant).

end should be avoided but recommended for C; (2) G and C at 3′ end of the primer is recommended. From the results of primer screening, it seems that RPA assay was tolerable to a wide range of primers with indistinct preference of the choice of nucleobase at 5′ end or 3′ end of the primer. Although no clear instruction is available to follow for RPA primer design leading repetitive tryouts among primer candidates, the assay has a high tolerability to primer design.[26] Overall, RPA assay is not complex in design compared to other isothermal amplification techniques (e.g., loop-mediated isothermal amplification, LAMP).[27]

The specificity of each RPA primer set was evaluated using 7 *S. enterica* strains and 7 non-*Salmonella* strains. The results are summarized in Table 2, and gel images are included in Figures S2 and S3. Due to high similarity in the sequence of the forward primers in each reverse primer group, one primer candidate per group was selected as an example of specificity evaluation. All *S. enterica* strains (i.e., *S.* Typhimurium, *S.* Enteritidis, and *S.* Typhi) were correctly identified by R21-F23 and R25-F26 primers, whereas all non-*Salmonella* strains (i.e., *Listeria*, *Arcobacter*, *Helicobacter*, etc.) generated clean negative results.

The sensitivity of each RPA primer pair was determined with 10-fold diluted *S.* Typhimurium concentrations ranging from 2 to 2.6 × 10^5 CFU/mL. The detection limit of the R21-F23 primer pair was 260 cfu/mL by both gel electrophoresis and LFD (Figure 3). LFD test line intensity was quantified as the percentage of the control line intensity. When bacterial concentration was lower than 260 CFU/mL, the test line intensity was not statistically different from that of the NTC. In comparison, the detection limit of the R25-F26 primer pair was 2.6 × 10^3 CFU/mL, as shown on both gel and LFD, which was 10-time less sensitive than that of R21-F23 assay. Thus, R21-F23 was selected as the optimal primer pair to detect *S. enterica* using the developed RPA assay. Sensitivity tests were repeated on the rest of primer sets (R21-F22, R21-F24, and R25-F27), in which R21-dependent primer sets resulted in a similar LOD as to R21-F23, while the R25-dependent primer set was the same as R25-F26 (data not shown). The reaction temperature was optimized in the range of 36−41 °C. The gel band was strong at temperature > 37 °C with the highest density at 40.6 °C (Figure S4). According to previous studies, the limit of detection (LOD) for *Salmonella* using RPA-based assays varied from 1 log to 2 log CFU/mL due to the difference in bacterial strains and primer selection.[28−30] Our developed RPA-LFD assay is specific and has comparable sensitivity as to that reported in a previous study.[28]

PES-Based Microfluidic "Lab-on-a-Chip" Device. The schematic illustration of the PES-based microfluidic device is shown in Figure 1. As an integrated microfluidic device, it aims to combine multiple functions including nucleic acid extraction, template amplification, and end-point detection on a single platform. There are three advantages of this microfluidic device developed in the current study. First, preparation and extraction of nucleic acids were maximumly simplified. Specifically, nucleic acids were released from bacterial culture under simple heat treatment for 10 min and a wax-printed paper-dipstick was used for DNA absorption and transportation.[16] The blank bottom area of the Whatman no. 1 filter paper dipstick was not wax-printed, thus being hydrophilic in favor of DNA binding, whereas the wax-printed handle was hydrophobic to prevent the unwanted wicking of

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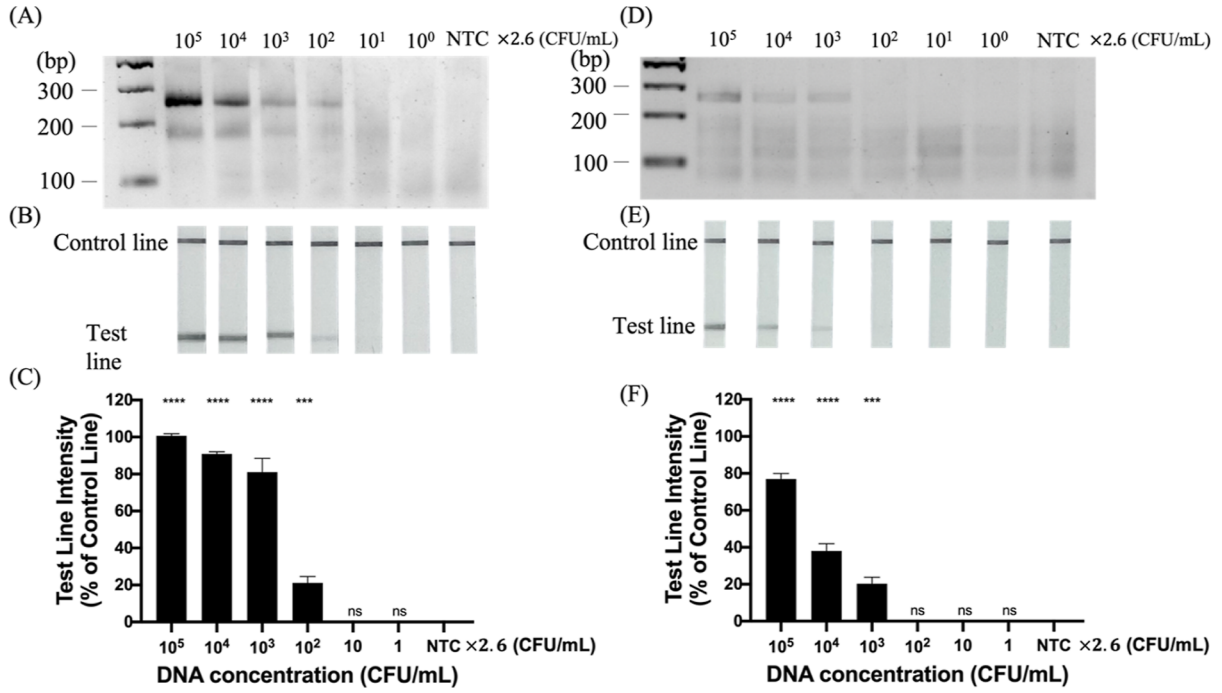

Figure 4. Performance of the RPA assay on PES-based microfluidic "lab-on-a-chip" device. (A–C) and chromatography paper (D–F). The sensitivity of the PES-based microfluidic device was 260 cfu/mL by both (A) 3% agarose gel electrophoresis and (B) LFD. The top line of the LFD is the control line, and the bottom line is the test line. The sensitivity of chromatography paper was 2.6×10^3 CFU/mL as examined by (D) 3% agarose gel electrophoresis and (E) LFD. (C,F) LFD test line intensity as the percentage of control line intensity (%) and statistically compared to the NTC (****$P < 0.0001$, ***$P < 0.001$, ns = not significant).

DNA mixture migrating from the bottom area. When paper-dipstick was dipped into the lysed bacterial culture, DNA was bound to the unwaxed bottom for the maximum capacity and transferred to PES membranes for RPA reaction. This novel DNA extraction was validated to maintain the sensitivity for the follow-up nucleic acid amplification,[16] and we obtained equal LOD by this approach as to reaction in the tube in the preliminary study (data not shown).

The second highlight was the use of the PES membrane instead of cellulose paper to carry out RPA reaction. The LOD of conducting RPA assay on the PES-based microfluidic device by both agarose gel electrophoresis and LFD was 260 CFU/mL (Figure 4), indicating equal sensitivity as to the reaction in the tube. The equal size of chromatography disc was inserted to replace the PES membrane for comparison. The LOD was 2.6×10^3 CFU/mL with fainter bands on the gel and decreased intensity on LFD. So far, most paper-based microfluidic devices use cellulose paper as the dominant scaffold material due to its ease on channel formatting and area blocking, as well as reagent release and protein binding capabilities. However, several studies also reported reduced isothermal amplification within the cellulose matrix.[15,16] Possible explanations include the structure of cellulose that hinders the diffusion of liquids and impedes the reagents from contacting with DNA template, especially after multiple layers of stacking. Recently, Linnes and co-workers discovered that the porous PES membrane allowed for efficient DNA and RNA amplification, as validated by both LAMP and HDA.[17] When a small amount of reagent was applied, cellulose filter paper absorbed liquid and failed to produce amplicons; in contrast, PES supported RPA reaction inside the membrane matrix. The mechanism of how reagent components managed to contact in the PES membrane requires further investigation, yet it is identified that the hydrophilic coating on the PES surface likely enhanced the performance of nucleic acid amplification.[17] The PES membrane also exhibits low non-specific protein absorption, but cellulose paper enables protein absorption. These characteristics likely interfere with compound diffusion and affect amplification efficiency. One drawback of PES is the lack of fabrication method to define hydrophilic and hydrophobic areas necessary to control liquid flow in the microfluidic device. To address this, we applied adhesive film as the background of the non-PES area, restricting a droplet size of space for RPA reaction on PES membranes. To further insulate RPA liquid droplet from the exterior environment (e.g., air and adhesive films), we may consider adding mineral oil in the reaction to form a hydrophobic outer shell, maximally confining compounds to react within the droplet and protect it from evaporation or cross-contamination during amplification.[31] In sum, we have validated the feasibility of RPA reaction on the PES membrane, which could be recognized as an appropriate material to design microfluidic devices for biomedical applications.

Last but not the least, we demonstrated fabrication simplicity of the PES membrane with adhesive film. Because current design involves no liquid flow, material (e.g., PDMS and cellulose paper) enabling the manufacture of inner liquid channels is not mandatory. This in return bypasses the considerations of fluid dynamics and technical complexity associated with soft lithography for PDMS (e.g., casting on molds and layer assembly) and requirement for an external pump.[10] Although a paper-based microfluid device overcomes these disadvantages, it still adversely impacts DNA amplification as mentioned above. Moreover, with the discontinuity of the Xerox wax printer, manufacture of the paper-based microfluidic device will be much more challenging. The

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concept of the currently developed microfluidic device was based on origami folding. Adhesive film was applied as a more desirable background to close two adjacent layers and excrete air in between. Three holes were cut at the boundary of layers 2 and 3 and layers 3 and 4 to ease the folding of two layers with minimum mechanical resistance. In this way, a well-designed folding device was established to support DNA amplification and secure sealed reaction space with the maximal simplicity.

Application of the PES-Based Microfluidic Device to Detect *Salmonella* in Agri-Foods. The performance of the PES-based microfluidic device for the detection of *S. enterica* was further validated using agri-food products including lettuce, milk, and raw chicken breast meat. Overnight *S.* Typhimurium culture was spiked into the decontaminated lettuce, raw chicken meat, and milk at the final concentration ranging from 3 to 3.2 × 10^3 CFU/mL, 4 to 4.4 × 10^3 CFU/mL, and 5 to 5.8 × 10^3 CFU/mL, respectively. After bacteria were rinsed off from food samples and enriched for 6 h, the PES-based microfluidic device was applied for detection. Both gel and LFD results for the detection of *Salmonella* in lettuce, chicken breast, and milk are shown in Figure 5. The PES-based microfluidic device was able to detect *Salmonella* in lettuce, chicken breast, and milk after 6 h enrichment at the concentration of 6 CFU/g, 9 CFU/g, and 58 CFU/mL, respectively.

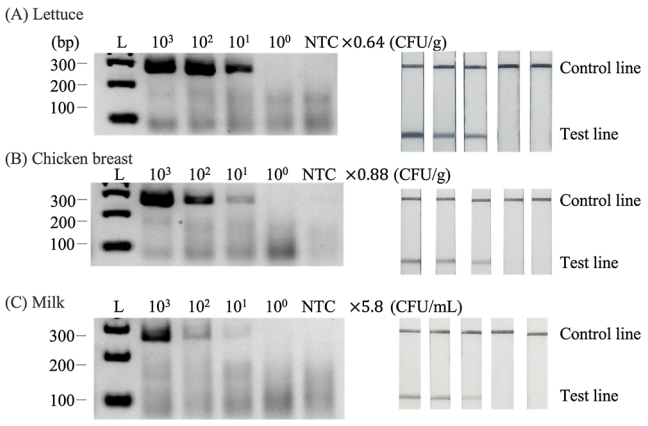

Figure 5. Detection of *S.* Typhimurium in spiked lettuce (A), chicken breast (B), and milk (C) after 6 h enrichment. The 10-fold serial dilutions of *S.* Typhimurium were spiked in lettuce, milk, and chicken, and the LODs were 6 CFU/g, 9 CFU/g, and 58 CFU/mL, respectively. DNA was extracted and RPA reaction was performed using the PES-based microfluidic "lab-on-a-chip" device. Results were examined by both 3% agarose gel electrophoresis and LFD. The top line of the LFD is the control line, and the bottom line is the test line.

Although all food samples with initial spiking concentration of ~2 log CFU/mL *Salmonella* were detected to be positive after 6 h enrichment, a slight difference of band intensities was observed among food commodities. Lettuce exhibited the strongest bands, followed by chicken breast meat and then milk with the weakest bands. LFD results agreed with that of gel images. According to a previous study, vegetable matrices such as tomato, cabbage, and broccoli have little effect on RPA reaction.[30] In another study, the same observation applied to lettuce where spiked lettuce was found slightly decreased sensitivity compared to that performed in tube without enrichment.[32] Chicken meat debris contain biological contaminants, such as blood, skin, and fats, and these might negatively affect RPA reaction.[33] However, the amplification efficiency of our microfluidic device on chicken meat was not largely reduced when compared to that on lettuce, which was probably due to the relatively pure bacterial culture with much less skin residues left after paper filtering. Similar results were obtained in a recent study where RPA was applied for the detection of *Salmonella* in chicken and broccoli, and the detection limit was comparable between these two food matrices.[34] Different from recovering DNA from lettuce and chicken that bacteria were washed off from food matrices and enriched in nutritious broth, *Salmonella* grew directly in milk in the current study, followed by cell lysis and DNA extraction for RPA detection. A recent study conducted by Kaur and co-workers reported that the LOD of *Salmonella* in milk by isothermal nucleic acid amplification (i.e., LAMP) was twice that of pure bacterial culture.[35] Different types of milk products also had various impacts on the performance of RPA assay. For example, milk (2% fat) had less negative effect on nucleic acid amplification than almond milk.[36] Specific compounds in milk (e.g., fat, protein, and calcium) that could impact DNA amplification in RPA assay remains largely unknown and requires further studies.

Although this RPA-based POC assay provided rapid and sensitive detection of *Salmonella* in food products, it is unable to differentiate viable bacteria from the dead counterparts due to the nature of RPA assay. Previous studies added intercalating dye (e.g., propidium monoazide, P) in PCR and LAMP reactions to differentiate viable and dead cells by inhibiting the amplification of double-strand DNA remained in the dead cells.[37,38] The feasibility of adding PMA into RPA to differentiate viable and dead *Salmonella* will be investigated in future studies.

CONCLUSIONS

We developed an integrated PES-based microfluidic "lab-on-a-chip" device for the detection of *Salmonella* from sample preparation to end-point detection via origami folding. The RPA assay showed 100% specificity to *S. enterica* against other bacteria. The PES membrane showed less interference to RPA than cellulose paper and exhibited equal sensitivity after multiple layers of stacking. The LOD was 260 CFU/mL in the tube and on the microfluidic device, while the chromatography paper-based counterpart displayed 10-fold less sensitivity. The performance of the PES-based microfluidic device to detect *Salmonella* was further validated using agri-food commodities. After 6 h enrichment, the device could detect *Salmonella* within 30 min at the concentration of 6 CFU/g in lettuce, 9 CFU/g in chicken breast meat, and 58 CFU/mL in milk. This innovative and cost-effective PES-based microfluidic device offered values in rapid detection of *Salmonella* and potentially other pathogenic bacteria in different sample matrices.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.3c00387.

Factory setting of the microfluidic "lab-on-a-chip" device, assembly of RPA reaction elements, specificity of the RPA assay, and optimization of the reaction temperature (PDF)

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Notes

The authors declare no competing financial interest.

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