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Multiplexed Detection of Pathogens Using Solid-Phase Loop-Mediated Isothermal Amplification on a Supercritical Angle Fluorescence Array for Point-of-Care Applications

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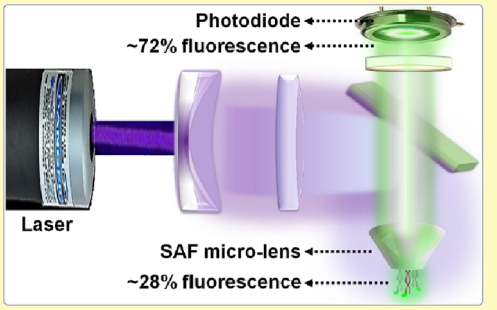
ABSTRACT: Adaptations of new generation molecular techniques for multiplexed detection of pathogens are gaining interest in the field of point-of-care (POC) industry and onsite testing. Loop-mediated isothermal amplification (LAMP), an advanced molecular amplification technique, has proven promising due to its unique features that suits ideal for POC applications. However, application of LAMP for multiplexed detection of pathogens remains challenging because of the difficulty in the identification of specific LAMP amplicons that does not have a well-definite molecular size. In this study, we developed a solid-phase loop-mediated isothermal amplification (SP-LAMP) technique to address the challenge. Integration of LAMP with the supercritical angle fluorescence (SAF) micro-optic structures as a solid support (SS) in an array format enabled spatial separation of LAMP amplicons in a multiplexed configuration. Important parameters such as length of the SS primers, length of the primer-binding region, the effect of surface density of immobilized SS primers, and cross-reactivity among the primers of different targets were iteratively tested and optimized. With the combination of SP-LAMP and SAF techniques, it was possible to detect multiple pathogens that include *Salmonella* spp, *Campylobater* spp., *Campylobacter coli*, *Campylobacter jejuni*, avian influenza virus (AIV), and pan avian internal control (IC) under singleplex conditions. The multiplexing capacity of the SP-LAMP was demonstrated using AIV and IC with promising results. The success of SP-LAMP has opened a promising direction toward the development of a multiplex POC system for rapid detection of multiple pathogens.



KEYWORDS: *loop-mediated isothermal amplification, point-of-care, multiplex detection, biosensor, solid-phase amplification, lab-on-a-chip*

Multiplexed detection of pathogens has become more important for both point-of-care (POC) diagnostics and for the rapid onsite screening of food products at industrial livestock production.[1−3] In the POC diagnostic industry, the molecular diagnostic test based on nucleic acid (NA) amplification is widely used.[4,5] At present, several commercial NA-based diagnostic systems for the multiplexed detection of pathogens are available such as i-STAT (Abbott), Piccoli Xpress (https://www.abaxis.com/piccolo-xpress), StatSensor, and StatSensor Epress (Nova Biomedical) (https://novabiomedical.com/uk/statstrip-creatinine/index.php), AQT90 FLEX (Radiometer) (https://www.radiometer.com/en/products/immunoassay-testing/aqt90-flex-immunoassay-analyzer), and PATHFAST (https://www.pathfast.eu/) (Table S1). However, the most challenging factors in the adaptation of these advanced molecular detection strategies in POC settings are the requirement of special equipment, stringent assay conditions, performance efficiency in minimal processed samples, and susceptibility to matrix inhibitors. In recent years, loop-mediated isothermal amplification (LAMP) has become more popular due to its robustness, simple to operate, high sensitivity, specificity, and more tolerance to inhibitors. LAMP is more ideal tool than conventional PCR for the integration into miniaturized POC devices[5−7] because of the following advantages: (1) in LAMP, the amplification occurs at a constant temperature between 60 and 65 °C that eliminates thermal cycling and relieves the complexity in the device development; (2) the unique feature of self-priming in the LAMP amplicons accelerates the reaction kinetics that reduces the analysis time; (3) the use of a special polymerase with high strand displacement activity provides LAMP a higher tolerance to PCR inhibitors; and (4) generation of an

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enormous amount of amplified products in the LAMP reaction opens the way for designing simplified instrumentations. However, the requirement of 4−6 primers with long sequences in the LAMP has limited its multiplexing capability.[5] The LAMP amplicons are complicated in their structure and lack a definite molecular size. Therefore, it is difficult to identify the target-specific amplicons in a pool of amplified products. A strategy of separating the LAMP amplicons is a prerequisite in order to adapt the technique to a multiplexed configuration. In general, there are two possible strategies to perform multiplexed analysis: (i) performing multiple singleplex reactions simultaneously in multiple chambers and[8,9] (ii) performing multiple reactions in an array format (microarray/DNA array) in a single reaction chamber.[10−12] The solid-phase (SP) amplification strategy performed in an array format, in which one or more primers are attached to a solid surface, is an example of a promising technique that has several advantages (please see the Supporting Information for details). This strategy results in the attachment of the amplified product to a well-defined two-dimensional area enabling spatial separation of different target-specific amplicons in a multiplexed configuration. Previously, the SP amplification strategy was demonstrated by our research group with the PCR technique (SP-PCR) using special micro-optic structures called super critical angle fluorescence (SAF) structures as solid support (SS) for amplification.[11−13] The use of SAF structures can improve the analytical sensitivity by enhancing the fluorescent collection efficiency. Emission of fluorescence from a fluorophore at or near the interface of two different materials of dissimilar refractive indices can result in the total internal reflection of the light. The radiation from a fluorophore on a surface is very asymmetrical where the light radiates mainly into the higher refractive index medium at a supercritical angle.[13] It was reported that ∼1 to 13 fluorophores (per μm^2) can be detected with truncated cone-shaped SAF structure, resulting in 36−40 folds increase in the sensitivity.[13] However, the application of the SAF microscopy concept was reported only with PCR. In this study, we report a SP isothermal amplification strategy as a novel technique by combining LAMP and SAF technologies. Here, the primers for the SP amplification are immobilized on top of the SAF structures embedded in an array format and amplified using LAMP. This novel concept was tested with *Salmonella*, *Campylobater* spp., *Campylobacter coli*, *Campylobacter jejuni*, avian influenza virus (AIV), and pan avian internal control (IC) with singleplex and multiplex detection conditions.

MATERIALS AND METHODS

Template DNA Preparation. *Salmonellaenteritidis*, *C. jejuni*, *C. coli*, and AIV were selected and used as target pathogens, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) avian gene was used as the pan avian IC for the development of the SP loop-mediated isothermal amplification (SP-LAMP) reaction. Bacterial strains *S. enteritidis* CCUG-32352, *C. jejuni* NTCC-11284, and *C. coli* CCUG-11283 were obtained from the culture collection of the National Food Institute, Technical University of Denmark. Genomic DNA of *S. enteritidis*, *C. jejuni*, and *C. coli* were isolated using the DNeasy blood and tissue kit (Qiagen, Germany) according to the instruction from the supplier. Nanodrop 1000 (Thermo Scientific, USA) was used to determine the DNA concentration. 2 ng/μL of genomic DNA was used as the template in the SP-LAMP reaction. For the AIV and the GAPDH IC, target genes were amplified by PCR using outer primers (F3 and B3) of respective LAMP primer sets, and the amplified products (10^{-6} dilution) were used as the template in the SP-LAMP reaction.

LAMP Primers and SS Primers of the SP-LAMP. In this study, six LAMP primer sets were used for the development of the SP-LAMP. The sequences of LAMP primers are shown in Table S2. Four primer sets targeting *Hil*A gene of *Salmonella* spp.,[14] 16S rRNA gene of *Campylobacter* spp.,[15] *guf*A gene of *C. coli*,[16] and oxidoreductase of *C. jejuni*[16] were selected from previous studies. Two primer sets targeting M gene of AIV and the GAPDH gene specific to the pan avian as IC (Reference no. NM_204305.1) were designed using software PrimerExplorer V5 (Eiken Chemical Co. Ltd., Tokyo, Japan). The primer sets for SP-LAMP of *Salmonella*, *Campylobacter*, *C. jejuni*, *C. coli*, and IC include F3, B3, FIP, BIP-cy3 (BIP primer labeled with cyanine-3 fluorophore at the 5′-end), and a modified FIP (ModFIP). The primer set for SP-LAMP of AIV consists of F3, B3, FIP-cy3, BIP, and a ModBIP primer. The ModFIP and ModBIP were designed by adding an oligo at the 5′-end of the FIP and the BIP primers, respectively. All the SS primers were coupled with a poly (T)10-poly (C)10 tail (10T10C tail). The SS primers were designed based on the inner primers (FIP or BIP). The binding regions of the SS primers were complementary to the loop regions of LAMP products called loop structures. The sequences of the SS primers are shown in Table S3. All the LAMP primers and SS primers were synthesized and purchased by LGC Biosearch Technology, Aarhus, Denmark.

The priming efficiency of conventional inner primer (BIP of AIV) and modified inner primer (ModBIP of AIV) was determined by performing LAMP experiments with target (cDNA of AIV) over a range of Log_5 concentration (0.16 pg to 2500 pg/reaction). Standard curves were generated, and isothermal doubling time (IDT) was estimated using eq 1[17,18] and the slopes of the respective standard graphs. The relative efficiency of LAMP with ModBIP was determined using eq 2.

$$\text{IDT} = -0.301 \times \text{slope of LAMP} \tag{1}$$

$$\text{Relative efficiency (\%)} = \frac{\text{slope (ModBIP LAMP)}}{\text{slope (BIP LAMP)}} \times 100 \tag{2}$$

Cyclic Olefin Copolymer Substrate for the SP-LAMP. Cyclic olefin copolymer (COC) slides (25 mm × 75 mm × 1 mm dimensions) and COC microchips were used as an inert SS in this SP-LAMP study. The microchip was designed with four chambers containing either 32 (8 × 4) or 35 (7 × 5) cone-shape supercritical angle fluorescence (SAF) structures in each chamber (Figure 1). Both the slides and the microchips were fabricated by injection molding (Engel Victory 80/45 Tech, York, PA) at National Centre for Nano Fabrication and Characterization, Technical University of Denmark (DTU Nanolab) using COC (Topas 5013-10, Topas Advanced Polymers GmbH, Germany). The fabrication of microchips was carried out, as described previously.[11] In short, a hard aluminum (alloy 2017, Metal Centret, Denmark) was used to mill a molding insert using a computer-controlled micromilling system (Folken Ind., Glendale, CA). The SAF structures in the molding insert were milled by using a 60° engraving tip (DIXI 7006, Le Locle, Switzerland). After polishing to get a smooth surface, the molding insert was inserted into the injection molding to fabricate the microchips.

Immobilization of SS Primers on COC Slides and Microchips. The SS primers were prepared in 0.004% Triton X-100 (Sigma-Aldrich, St, Louis, MO) and 5× saline sodium citrate (SSC) buffer (Promega, Madison, WI) at a final concentration of 60 μM. The SS primer solutions were spotted on cleaned COC slides using Nano-Plotter (Analytik, UK) with approximately 600 pL/drop and on COC microchips using a non-contact array system (sciFLEXARRAYER S5, Scienion AG, Berlin, Germany) with a PDC coating type 1 spotting tip, that could deposit 300−360 pL/drop. After spotting, the slides and the microchips were dried at room temperature and exposed to UV irradiation at 254 nm with a power of 3 mW/cm^2 for 10 min in an UV cross-linker (Stratalinker 2400, Stratagene, CA, USA). Subsequently, the slides and microchips were washed with 0.1× SSC for 5 min, rinsed with Milli-Q water, and dried at room

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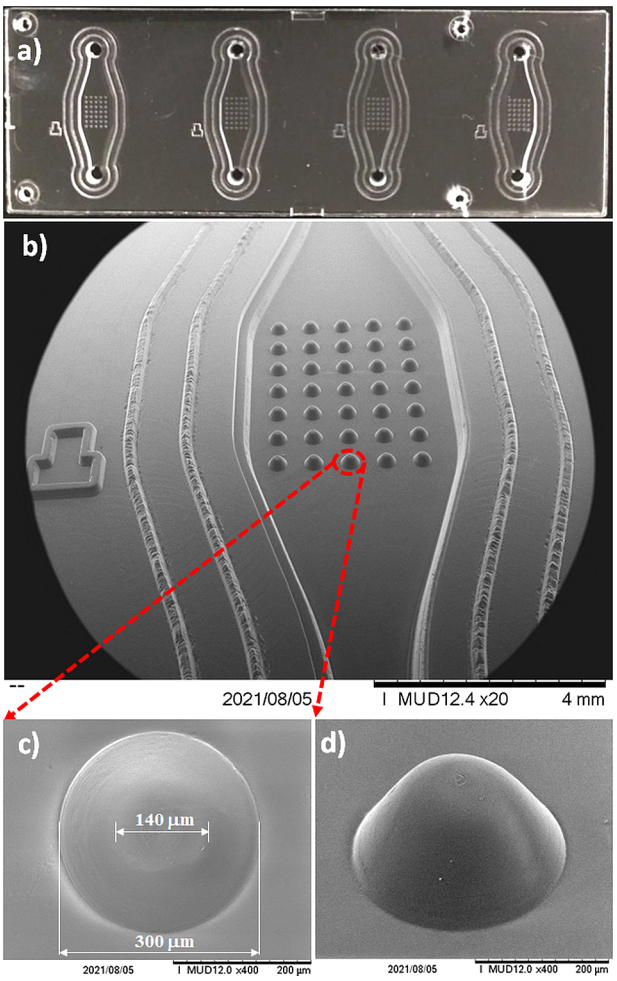

Figure 1. (a) Polymer microchip with four reaction chambers, (b) SAF embedded as an array in the microchip, and (c,d) closer view of the SAF micro-optic structure.

temperature. After drying, both COC slides and the microchip chambers were treated with bovine serum albumin (2.5 mg/mL) for 30 min at room temperature. Furthermore, the slides and microchips were rinsed with Milli-Q water, followed by drying at room temperature. On the slide, a gene frame (Life Technologies Europe BV, Denmark) was attached to cover the area spotted with SS primers that formed a reaction chamber with a liquid holding capacity of 25 μL, and the SP-LAMP reaction was performed. Microchips embedded with SAF structures were sealed with a transparent plastic foil on the top using ultrasonic welding (Telsonic USP4700 20 kHz ultrasonic welder, Telsonic, Erlangen, Germany). The microchip was mounted in a custom texture followed by aligning a foil on the top. The foil was welded by applying 2 bar welding pressure at an energy of 20 W/s and 80% vibrational amplitude. The average time of the welding process was around 30 s per chip.

SP-LAMP Reaction. Singleplex SP-LAMP assay was carried out using 25 μL of master mixture containing target-specific primers (the concentration of primers used for each target is shown in Table S2), 1.4 mM dNTPs mix (LGC Biosearch Technology, Aarhus, Denmark), 0.5 M Betaine (Sigma-Aldrich, Denmark), 10 U Bst 2.0 Warmstart DNA polymerase (New England BioLabs), 1× isothermal amplification buffer, sterilized water, and DNA template. On the slide, the SP-LAMP master mix was pipetted into the gene frame and sealed using a coverslip. On the microchip, the SP-LAMP mixture was loaded through an inlet hole into the chamber and sealed using a thermostable, PCR-compatible optical adhesive cover (Applied Biosystems, Waltham, MA).

The SP-LAMP assays were conducted in a flatbed PCR thermocycler (MJ Research, Canada). The SP-LAMP reactions were set up at 65 °C for 90 min. After completion of the reactions, the solutions inside the gene frames and chambers were collected for post-amplification analysis by gel electrophoresis. The slides and chambers in microchips were washed with 1× SSC, followed by washing with 1× SSC containing 1% Tween-20 for 10 s, and two times with Milli-Q water for 20 s. After washing, the slides and microchips were dried at room temperature. For visualization of SP-LAMP results, the microchips were scanned by the Cy3 filter at 200 ms exposer time in a BioAnalyzer 4F/4S scanner (LaVision BioTec GmbH, Bielefeld, Germany).

Multiplex SP-LAMP Reaction. The multiplex SP-LAMP was tested initially with 11 different combinations of two primer sets targeting (1) *Campylobacter* spp. and *Salmonella* spp., (2) *C. coli* and *C. jejuni*, (3) *Campylobacter* spp. and *C. coli*, (4) *Campylobacter* spp. and *C. jejuni*, (5) *Salmonella* spp. and *C. coli*, (6) *Salmonella* spp. and *C. jejuni*, (7) *Campylobacter* spp. and IC, (8) *Salmonella* spp. and IC, (9) *C. jejuni* and IC, (10) *C. coli* and IC, and (11) AIV and IC. The conditions of the multiplex SP-LAMP reaction were similar to the conditions in the singleplex SP-LAMP reaction, except two primer sets were used. The multiplex LAMP reaction was initially studied in the liquid phase (LP) (without the attachment of SS primers on the solid substrate) in tubes using a thermocycler (Mx3005P, Stratagene, AH diagnostics, Denmark) at 65 °C for 90 min. The multiplex SP-LAMP on microchips was performed with the attachment of SS primers similar to singleplex SP-LAMP, as described above.

Effect of SS Primer Concentrations on SP-LAMP Assay. Different concentrations of the SS primer targeting *Hil*A gene of *Salmonella*, ranging from 10 to 60 μM, were spotted on the COC slide by NanoPlotter with approximately 600 pL per droplet. The immobilization process and the SP-LAMP reaction were carried out, as described above. After the SP-LAMP reaction, the slide was scanned by GenePix Pro 4200A, (Molecular Devices) with 500 gain.

Data Analysis. The scanned image was analyzed using GenePix Pro 7.0 software (Molecular Devices). A circle was drawn and adjusted to the size of the spot. The mean fluorescence signal intensity value inside the circle was determined as the signal intensity of the spot. On the slide, the intensity of fluorescence signals of an area between the spot and the area of the slide that was not immobilized with SS primers was used as the background. The standard deviations of the background were calculated based on the values of the background signals from five different areas. There were two backgrounds in the microchip. Background signal 1 was the mean value of the signals from five areas in the microchip without SAF structures. Background signal 2 was the mean value of the signal of five SAF structures without any immobilized SS primers. The signal of background 1 subtracted from signal of background 2 was considered as the background of the microchip. The signal-to-noise ratio (SNR) (eq 3) was determined by the mean of the intensity of the fluorescence signals ($\bar{\mathbf{F}}_\mathbf{i}$) of the five spots (in the slide) or seven SAF structures (in the microchips) after amplification, minus the mean background ($\bar{\mathbf{B}}$), and divided by the standard deviations of the background ($\mathbf{SD}_{\bar{\mathbf{B}}}$).

$$\mathbf{SNR} = (\bar{\mathbf{F}}_\mathbf{i} - \bar{\mathbf{B}})/\mathbf{SD}_{\bar{\mathbf{B}}} \quad (3)$$

Gel Electrophoretic Analysis. After the SP-LAMP, the reaction mixtures were collected and confirmed by gel electrophoresis. 8 μL of each LAMP amplified product was loaded on agarose gel (2%) containing 1× SYBR Safe DNA gel stain (Invitrogen, Life Technologies, USA). The gel electrophoresis was carried out at 50 Volts for 30 min and was visual under UV light using a BioSpectrum AC imaging system (AH diagnostics, Denmark).

RESULTS AND DISCUSSION

Solid-Phase LAMP (SP-LAMP) Process. SP-LAMP is the amplification of a target NA on an inert solid surface under isothermal conditions. The dsDNA generated in SP-LAMP will eventually be localized on the solid surface induced by the

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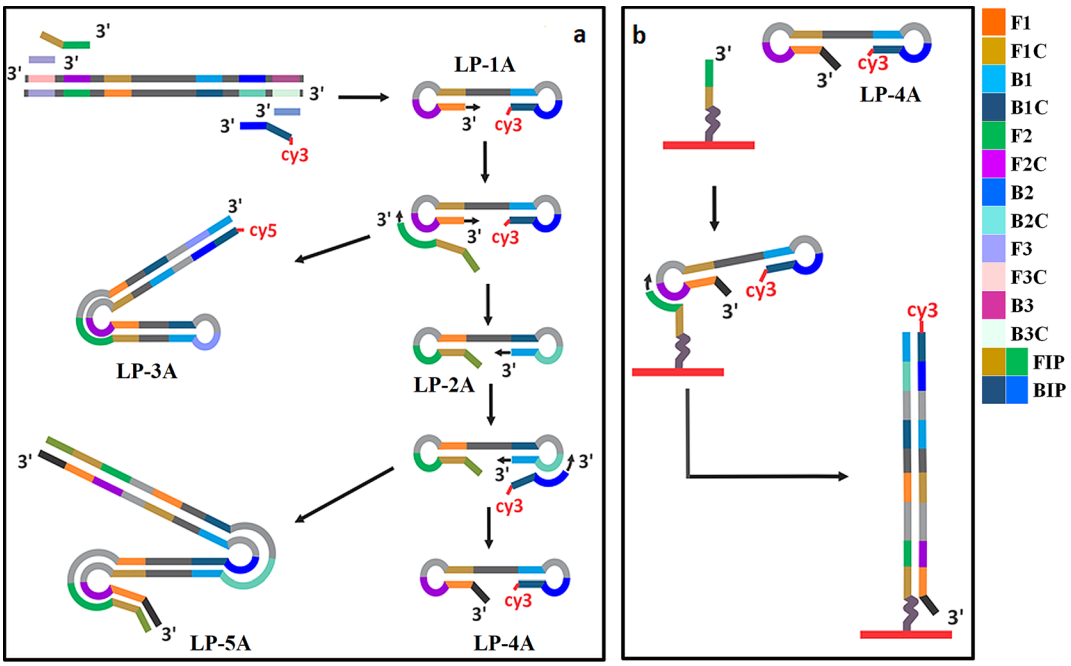

Figure 2. Schematic representation of the SP-LAMP reaction. The SP-LAMP includes two phases of amplification: (a) LP amplification and (b) SP amplification.

immobilized SS primers (Figure 2). The SP-LAMP comprises two main amplification steps: LP (Figure 2a) and solid phase (SP) (Figure 2b). In the initial LP amplification step, a loop structure (LP-1A) is generated by the combined activities of two outer primers (F3 and B3) and two inner primers (FIP and BIP-cy3). This loop structure contains a self-priming region at the 3′-end as conventional LAMP and a fluorescence dye (cy3) at the 5′-end. Similar to conventional LAMP, the loop regions are the positions for primer (FIP and BIP) binding that generates new strands in subsequent amplification cycles. In addition, in the LP, the modified primer (either ModFIP or ModBIP as it is designed for assay) binds to the F2C (or B2C in the case of ModBIP) region on LP-1A and synthesizes a complementary (LP-1B) strand, leading to the release of two other structures LP-2A and LB-3A. This is followed by continuous annealing of BIP-cy3 to LP-2A at the B2C region and extends a new strand, thereby releasing LP-4A and LP-5A structures simultaneously. The loop structure LP-4A contains a fluorescent dye at the 5′-end but not a self-priming region at the 3′-end. This structure acts as a template for SP amplification. In the second step, the F2 region of SS primer immobilized on the solid substrate anneals to the F2C region on LP-4A and synthesizes complementary strand. As a result, a dsDNA labeled with the fluorescence dye cy3 is generated on the solid surface.

The priming efficiency of the modified inner primer was assessed based on the IDT with ModBIP of AIV as an example. Unlike PCR, LAMP does not have the cyclical constraint, and therefore, conventional calculation methods do not provide accurate amplification efficiency values. LAMP is a continuous process that results in a growing concatenation of amplicons rather than discrete individual copies over well-defined control cycles. Therefore, performance of LAMP (BIP v/s ModBIP of AIV as an example) was compared through a unique metric called IDT. Analysis was based on the assumption that LAMP reactions resulted in exponential rates of DNA polymerization corresponded to the time when a constant repeatable threshold quantity of dsDNA was produced.[17] IDT is the time required by different starting quantities of template to reach a uniform threshold[17,18] (IDT = −0.301 m, whereby 0.301 corresponds on the base 10 logarithmic scale to a change in the concentration of a factor of 2 and m = slope of a standard curve). The LAMP with the conventional approach (BIP) had an IDT of 1.9 min (Figures S1a and S2), whereas LAMP with modified inner primer (ModBIP) had an IDT of 2.33 min (Figures S1b and S2). The sensitivity of LAMP with ModBIP was ∼5 times lower (∼0.5 to 1 pg of cDNA of AIV) compared to the conventional LAMP with BIP (0.1 to 0.2 pg of cDNA of AIV). The relative efficiency of LAMP with ModBIP was ∼78.47% according to their respective slopes (Figure S2). In the SP-LAMP, the modified inner primer generates a shorter product (LP-4A, Figure 2). Because of the modification, the extension of LP-4A terminates due to non-complementary oligo tail at its 3′-end. In the SP-LAMP reaction of AIV, this has resulted in a product having size of ∼218 bases with two loop structures on either end. However, the conventional inner primer (BIP without any modifications) generally results in a product with larger size (LP-3A, Figure 2). LP-3A continues with an unrestricted extension at its 3′-end forming eventually a concatemeric DNA molecule with multiple copies of the same sequence. To confirm this, the LAMP products were assessed through agarose gel electrophoresis after reaction termination, and the intensity of respective amplicons were quantified through image analysis (Figure S3). It was found that the concentration of LP-4A resulted with ModBIP was 20.7% more than LP-3A (product of BIP, conventional LAMP). In principle, LP-4A is the preferred primary product that further acts as a template for the SP reaction.

In the present study, SAF structures facilitated SP amplification as an inert support. The SAF structures were embedded in the reaction chamber of the COC microchip wherein the chamber had a depth of ∼500 μm with a holding capacity for 25 μL reaction solution. These SAF structures were around 140 and 310 μm wider at the top surface and at

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the bottom, respectively, with mean surface roughness of ~161 nm (Figure 1). The SAF structures were embedded with an inter distance of 0.625 μm between the structures.[11] The SAF structures were truncated with conical shaped transparent polymer structures that also acted as a micro-optic lens. The SAF structures were able to collect ~31 fold more fluorescence signals due to their superior fluorescence collection efficiency. Previously, it was reported that the collection of the fluorescence signal from the SAF array is almost independent of the numerical aperture (NA).[13] It is worth mentioning here that the distribution of fluorescence emission from a surface-bound fluorescent molecule is anisotropic. This asymmetrical phenomenon directs the fluorescence lights to radiate more toward the higher refractive index medium such as a polymer surface[10,13] at the angle larger than the critical angle of the two media. The polymeric SAF microlens has higher refractive index (n = 1.53) compared to air (n ~ 1) or water (n = 1.33). It was reported that the fluorescence collection efficiency in the low refractive medium (air) is proportional to the NA of the optical system (~NA × $\sin\theta_1$) (where θ_1 is the collection angle of the objective), due to the approximately even distribution of the emission in all directions. In the high refractive medium (n = 1.53), the SAF structures collect the fluorescent light within the angle range from 40.8 to 79.2° due to the total internal reflection at the sidewalls of the SAF structures. It was also reported that about 98% of the total fluorescent light emitted into the SAF was collected.[10,13] As a result, the limit of detection of a fluorescence signal achieved with the SAF structure was ~7 nM that corresponds to ~46 copies of fluorophore-labeled oligonucleotide probe/μm^2 area (Figure S4) considering ~50% immobilization efficiency. Stability of the immobilized SP primers on the SAF in the microchip was assessed based on the retention of the fluorescence of spotting control probes (Figure S5). The fluorescence signals from the spotting control probe of three different microchips were recorded on day 1, after 3 weeks, after 1 year, and after 2.5 years. In each chip, minimum 5−6 SAF structures were analyzed, and average fluorescence signals were recorded for calculations. It was observed that the immobilized probes were stable for almost a year when stored under dry conditions without any special treatment. The SNR remained same after 3 weeks, and there was ~16% decrease in the SNR after 1 year. The SNR reduced almost 65% after 2.5 years of storage (Figure S5).

Evaluation of SS Primers for SP-LAMP. To evaluate the efficiency of SS primers for the SP-LAMP reaction, three SS primers (FIP, FIP21, and FIP22 SS primers) that are different in their length at the binding region were designed based on the FIP primer for specific detection of *Salmonella* spp. (Figure 3). The binding region of the FIP SS primer was F2 (21 bases), while the FIP21 SS primer was 36 bases (F2 plus additional 15 bases), and the FIP22 SS primer was 46 bases (F2 plus 25 bases). All the SS primers used in this study contained a 10T10C tail at the 5′-end that forms a covalent bond with unmodified COC surface during UV treatment. Figure 3b shows that the SS primers with longer binding sequence have better SP-LAMP efficiency than that of the shorter SS primers. The results were consistent with the previous reports.[19−21] The longer SS primers might have a higher probability of binding to the target than the shorter SS primers resulting, in general, in higher sensitivity than that of the shorter one.[20] In addition, the longer SS primers could extend farther away from the solid surface, and therefore, the

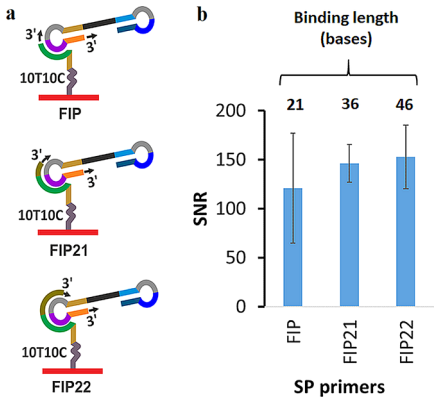

Figure 3. Evaluation of efficiency of SS primers for SP-LAMP. (a) Design of SS primers and (b) efficiency of SP-LAMP using different SS primers.

longer SS primers are easier to anneal to the DNA target on the LP.[19] In order to evaluate the hybridization efficiency of SS primers, the thermodynamic properties of both conventional and modified inner primers of AIV and IC were assessed using the UNAFold web server (http://www.unafold.org/). The two state melting hybridization temperature and Giggs free energy (ΔG) values were compared under the settings of the hybridization conditions similar to LAMP assay conditions at 65 °C. There were no significant differences observed in the Giggs free energy (ΔG) of conventional and modified inner primers (Table S4). Further analysis of the melting temperature (T_m) of the hybridized dsDNA product of each primer with its respective LP-4A complementary template (loop product of the modified inner primer) revealed highly stable dsDNA with the modified inner primer even at the LAMP reaction temperature (65 °C). The melting temperature of the hybridized dsDNA product of each SP-primer with its respective complementary LP-4A was >80 °C. Under multiplexed condition, LP-4A of the IC target (loop product of ModFIP) was analyzed with AIV primers, and LP-4A of the AIV target (loop product of ModBIP) was analyzed with IC primers as non-specific or non-complementary targets. The melting temperature of SP-primer with non-specific target was 25.5 °C for AIV and was 11.1 °C for IC SP-primer (Table S4).

Effect of the SS Primer Concentration on the SP-LAMP. In the SP amplification, density of the surface-bound oligonucleotides plays an important role in determining the thermodynamics of reaction kinetics and DNA hybridization.[22] To investigate the effect of SS primer concentrations in the SP-LAMP reaction, different concentrations of the FIP SS primer ranging from 10 to 60 μM were initially tested on COC slides. Since the UV immobilization method has an efficiency around 50%,[21] the actual surface density of the immobilized SS primers may vary from 2.48 × 10^{10} to 1.49 × 10^{11} molecules/mm^2. Figure S6 shows that the SNR was higher when the higher concentration of the SS primer was used. Previous reports suggested that an optimal SNR could be obtained with surface-bound oligonucleotides density of ~10^7 to 10^8 molecules/μm^2.[21,23] At lower concentrations of the SS primers, the efficiency of the SP DNA amplification decreases because of the incomplete reaction.[24] At higher concentrations, the efficiency of surface-mediated amplification decreases with increasing density of the surface-bound oligonucleotide probes.[25] Peterson et al. reported that, at higher density of SS primers, only 10% of SS primers were utilized for

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hybridization; thus, the binding kinetic will be lower.[24] This effect might also affect the efficiency of SP-LAMP. The loss of the amplification efficiency could be due to several factors such as (i) masking effect wherein, the longer amplicons could potentially mask adjacent primers that are within their one gyration radius. (ii) Molecular crowding effect resulted by molecular repulsion due to increased steric hindrance between the SP primers immobilized within the radius of gyration. (iii) The Coulombic blockade effect due to the dominance of electrostatic repulsive forces over the hybridization that pushes the free-floating template to move away from the surface.[23] In the SP-LAMP, the loop structures of the primary amplification products (LP-4A, Figure 2b) are used as the template during the exponential amplification cycle. At higher density, the binding of this loop structure to the SS primers may get affected because of poor diffusion kinetics and lack of molecular accessibility. Therefore, one of the recommendations to improve the SP amplification is to suppress the Coulombic repulsion through surface modifications with multivalent counterions or by enhancing positive electrostatic potential at the surface.[23] Another recommendation is to characterize the surface adsorption conditions of those systems relying on surface-mediated hybridization.

SP-LAMP for Singleplex Detection of Pathogens. SP-LAMP was evaluated at singleplex detection condition with different pathogens that include *Campylobacter* spp., *C. jejuni*, *C. coli*, AIV, and IC. The SS primers of *Campylobacter* spp., *C. jejuni*, *C. coli*, and IC were designed based on FIP primers, and respective BIP primers were labeled at their 5′-end with Cy3 fluorescent dye. In contrast, the SS primers of AIV were designed based on the BIP primer, and the FIP primer was labeled with Cy3 at the 5′-end. For each target, several SS primers having different lengths at their binding region were designed and tested. We investigated the SP-LAMP on COC microchips containing SAF structures. Performing SP-LAMP on SAF structures was advantageous as it minimizes the undesirable interactions between the SP-LAMP amplicons since the amplicons were separated spatially on the solid surface. The control probes (Ctr) gave strong signals in all the SP-LAMP reactions (*Campylobacter* spp., *C. coli*, *C. jejuni*, IC, and AIV), indicating a good immobilization of SS primers on SAF structures (Figure 5a). The SP-LAMP amplifications were positive for all the targets in this study. However, the efficiency of SP-LAMP was different between the SS primers and targets (Figure 4). For each target, the SS primers having a longer binding region gave higher SNR than that of the shorter SS primer (Figure 4). Of the four targets tested, the AIV SS primer (AIV3 and AIV4) gave the highest SNR (from 158 to 163), followed by IC (IC3, SNR 153), *C. coli* (Cc2, SNR 104), and *Campylobacter* spp. (Cs3, SNR 59). The SS primers for *C. jejuni* had the lowest SNR in all the three SS primers (from 6 to 21) (Figure 4).

The efficiency of SP-LAMP might be affected by the length of the binding region of SS primers.[19−21] The SS primers with a long binding region would facilitate better hybridization with the template in the LP reaction. Therefore, we expected that all the SS primers with longer binding sequences would give a better signal than shorter ones. As expected, for each target, the SS primers with the longer binding region gave better SNR than the shorter (Figure 4). However, this trend was not followed by some of the SS primers in different targets, for example, SNR of the AIV4 (60 bases in the binding region) and IC3 (53 bases in the binding region) was better than that

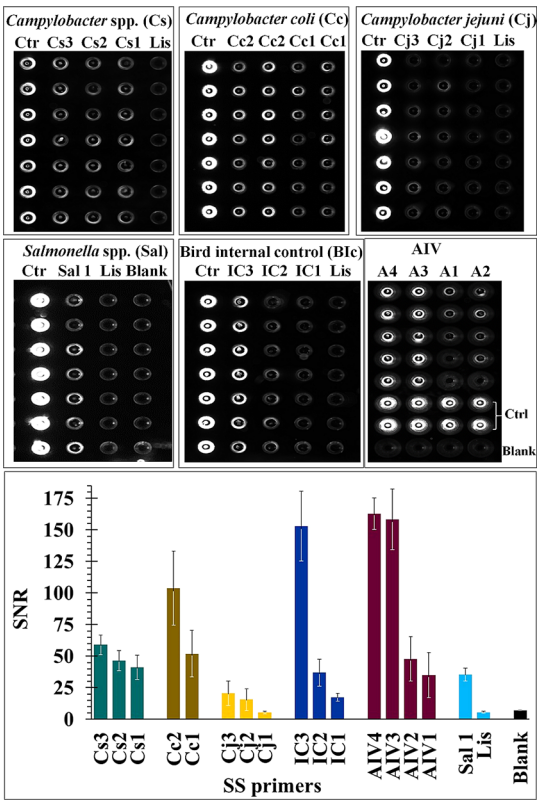

Figure 4. Efficiency of SS primers in SP-LAMP tested with different targets.

of Cc2 (43 bases in the binding region), Cs3 (74 bases in the binding region), and Cj3 (51 bases in the binding region). These differences could be due to the overall efficiency of SP-LAMP primer sets of individual targets. The difference in the turnover rate of the individual LAMP reaction resulting in the formation of primary loop structures might have significantly influenced the outcome in the SP-LAMP reaction. Another reason could probably be the dominance of LP reaction over the SP reaction among the different targets. In general, the dynamics between the initial hybridization of free-floating primer and surface-immobilized primer with their DNA templates differ substantially. The mechanism of surface-mediated hybridization is slightly complex compare to LP hybridization. In principle, surface-mediated DNA amplification relies on the thermodynamic equilibrium between hybridized and unhybridized state of DNA templates with accessible primers. Accessibility of free-floating primers is higher than the stationary form of the primers (due to immobilization) if both forms are at similar concentrations. In the SP-LAMP, this thermodynamic equilibrium relies on the hybridization of loop structure (LP-4A, Figure 2) between free-floating FIP, modified FIP (FIP with an added oligo at the 5′-end) in the LP, and surface-bound FIP (FIP immobilized on the SAF structure). Classical models that describe the rate of surface-mediated hybridization have suggested "specific adsorption" (direct adsorption of DNA templates to immobilized primers prior to hybridization) and "nonspecific adsorption" (initial adsorption of DNA templates to the surface induced by diffusion of the DNA templates followed by subsequent hybridization) as the two probable mechanisms.[25,26] Therefore, lack of diffusion of templates results in inefficient priming and subsequently inefficient reaction.[27] In

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the recent past, researchers have adapted several approaches to enhance the diffusion of templates into the solid surface: (i) supplementing the LP reaction with unbound primers to increases the copy numbers of the starting template.[28] However, this may results in higher background and accumulation of undesired byproducts. (ii) Reducing the concentration of LP primer, as an asymmetric approach, to reduce the competition between the aqueous and the SP primers during the annealing and extension steps.[6] (iii) Increasing the melting temperature of SP primer and increasing the annealing temperature at a later stage of the amplification to favor SP priming.[29]

Development of SP-LAMP for Multiplex Detection of Pathogens. In SP-LAMP, five primers are required per target. For multiplexing SP-LAMP, for two targets, at least 10 primers are required per reaction. Under such circumstances, the risk of the formation of heterodimers among primers is high due to the long sequences of primers. To determine the possibilities of primer−dimer interactions, a multiplex SP-LAMP involving two different targets was first tested without any target in the LP. DNA intercalating dye, SYTO 9, was used to monitor the signals being generated due to the intercalation to primer dimers. The signal intensity was normalized to the signal intensity observed in a positive reaction. Since the dye does not fluoresce in the absence of dsDNA and emit fluorescence strongly in the presence of dsDNA, the normalized signal (%) is presented to compare the primer dimers of singleplex and multiplex targets (Figure S7). In this test, the background fluorescence signal of the SYTO 9 without any target was used to compare with signals of single target and multiple targets. In the mixture containing all LAMP reagents except primers and target, the background fluorescence signal of SYTO 9 was ∼9% of the positive reaction (Figure S7). In the presence of the singleplex primer set, the background fluorescence signal of SYTO 9 was 11−12% for IC, AIV, and *C. jejuni*; and 22−23% for *C. coli*, *Salmonella*, and *Campylobacter* in comparison to the positive reaction (Figure S7). With the primer sets for multiple targets, the fluorescence signal of SYTO 9 was 12% for *C. jejuni* and IC, 14% for AIV and IC, 22% for *C. coli* and IC, 23% for *C. jejuni* and *Salmonella*, *C. coli* and *C. jejuni*, and *Salmonella* and IC, 25% for *C. jejuni* and *Campylobacter*, 27% for *C. coli* and *Salmonella*; 29% for *Salmonella* and *Campylobacter*, and 31% for *C. coli* and *Campylobacter*. The fluorescence signals for these pairs were not significantly higher in comparison to their respective singleplex targets. The results indicated a low number of heterodimer of primers in each combination. In contrast, the fluorescence signal was increased remarkably up to 75% in the multiplex of *Campylobacter* and IC combination, and the normalized signal was 50% higher than their singleplex targets (Figure S7). The fluorescence signals observed in this combination indicated the high heterodimer of different primers between *Campylobacter* and IC primers. For further investigation of the SP-LAMP multiplexing capacity, two combinations of targets that showed the lowest heterodimer (the AIV/IC, and the *C. jejuni*/IC) were selected and tested in the presence of SS primers corresponding to their targets. There were no significant differences observed in the background fluorescence of SYTO 9 between singleplex and multiplex primer sets of both targets (Figure S8). The signal differences were lesser than 1−2%.

The primer dimers are a common issue in LAMP because of a high number of primers being used with longer sequences in the reaction. The primer dimer (both homodimer and heterodimer) in singleplex reactions could be minimized by using LAMP primer design software (Eiken Chemical Co. Ltd., Tokyo, Japan). The heterodimer of primers in the multiplex reaction (multiple primer sets) could adversely affect the multiplex LAMP reactions. The primer dimers may get amplified if primers hybridize at their 3′-end, resulting in false positive results.[30] Besides, the amplification efficiency of the SP-LAMP reaction might be reduced by the heterodimers. Therefore, it is important to check the primer dimers of different LAMP primer sets for multiplexing SP-LAMP. All the combinations for duplex targets tested in this study did not result in any false positive reactions.

The SP-LAMP for multiplex detection of AIV and IC was further tested on COC microchips (Figure 5). In the negative

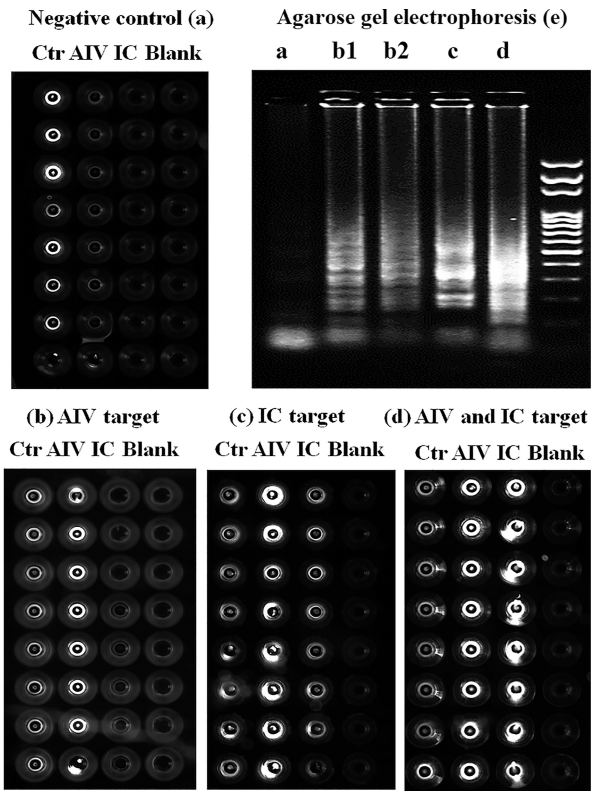

Figure 5. SP-LAMP for multiplex detection of AIV and IC. (a) Negative control with no targets, (b) only with AIV target, (c) only with IC target, (d) with both AIV and IC targets together, (e) agarose gel electrophoresis of reaction solution of the SP-LAMP experiment of negative control with no targets (a), only with AIV target (b1 and b2 are repetitions of the same experiments), only with IC target (c), with both AIV and IC targets together (d).

control reaction (Figure 5a), no fluorescence signal was observed with both AIV and IC SS primers that confirmed the specificity of the multiplex SP-LAMP reaction. The fluorescence signal was observed with AIV target (Figure 5b), with IC target (Figure 5c), and with both AIV and IC targets (Figure 5d) that confirmed the amplification of specific targets in the multiplex reaction of AIV and IC. However, the false positive signal was also observed with AIV SS primers in the SP-LAMP reaction with the IC target (Figure 5b). The false positive signal might be due to the non-specific amplification of the AIV SS primer with IC product generated during the SP-LAMP reaction. It was reported that the SS primers with a longer binding region gave better sensitivity; however, they had

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lower specificity than the shorter SS primers.[19] The AIV3 SS primer used in this multiplex detection contained 55 bases (F2 plus 33 bases) in the binding region. The specificity of the F2 region was confirmed during the development and optimization of conventional LAMP for AIV but not for the structure with 33 bases extension. This 33 bases extension in the binding region might be the reason behind non-specific binding with IC product. However, the thermodynamic analysis of AIV3 and IC SS primers under multiplexed condition (parameters set at 65 °C, 50 mM K^+, and 2 mM Mg^{2+}) predicted that the melting temperature of the AIV SS primer with LP-4A of the IC target (non-specific hybridization) was 25.5 °C, and the melting temperature of the IC SS primer with LP-4A of AIV target was 11.1 °C (Table S4). Under conventional experimental design, the unmodified inner primers (being used in the LP reaction) also have higher melting temperatures (>75 °C) for their target-specific hybridized products (Table S4). This produces enormous amount of unwanted DNA products. Suppression of such unintended reactions could be a strategy to overcome false positive signals in future.

CONCLUSIONS

In brief, this report details the development of SP-LAMP assay as a novel technology for the detection of multiple pathogens. The performance of assay was successfully tested with *Campylobacter* spp., *Salmonella* spp., *C. coli*, *C. jejuni*, AIV, and IC in singleplex conditions. Furthermore, the technique was also tested to extend the functional capability of the SP-LAMP technology and demonstrated multiplexing SP-LAMP with AIV and IC targets. Initial studies with multiplexing SP-LAMP in this study gave promising results, although the specificity of the SP amplification still remains a challenge. The multiplexing capacity of LAMP itself is not yet completely developed and, therefore, is one of the major research fields in the contemporary scientific world. In this direction, initial promising results observed in this study for the SP-LAMP opens the way toward possible success in the near future. Finally, (i) an asymmetric approach to reduce the competition between the aqueous and the SP primers in combination with (ii) elevated melting temperature of SP primer, (iii) stepwise amplification by increasing the annealing temperature at a later stage of the amplification, (iv) suppressing priming efficiency of unmodified inner primers thereby reducing the generation of unwanted DNA products, and (v) proper characterization of the adsorption conditions of surface and blocking unwanted non-specific adsorption of negatively charged NAs on the polymer surfaces are recommended to improve the performance of multiplexed SP amplification.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.2c01337.

> Current techniques available/reported for the detection of pathogens, details of sequences of probes and primers used in this work for bacterial detection, analysis of thermodynamic properties of SP primers, additional data on SAF characterization, stability of immobilized probes, and SP-LAMP optimization (PDF)

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Author Contributions

T.L.Q. and A.C.V. equally contributed to this work and should be considered as equal first authors. The manuscript was written through the contribution of all authors. A.W., D.D.B., T.L.Q., A.C.V., and M.G. discussed and designed the work. T.L.Q. and M.G. performed the experiments. T.N. and H.V.N. provided slides design and fabricated the microchips. A.C.V. and T.L.Q. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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