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An integrated lateral flow assay for effective DNA amplification and detection at the point of care†

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Lateral flow assays (LFAs) have been extensively explored in nucleic acid testing (NAT) for medical diagnostics, food safety analysis and environmental monitoring. However, the amount of target nucleic acid in a raw sample is usually too low to be directly detected by LFAs, necessitating the process of amplification. Even though cost-effective paper-based amplification techniques have been introduced, they have always been separately performed from LFAs, hence increasing the risk of reagent loss and cross-contaminations. To date, integrating paper-based nucleic acid amplification into colorimetric LFA in a simple, portable and cost-effective manner has not been introduced. Herein, we developed an integrated LFA with the aid of a specially designed handheld battery-powered system for effective amplification and detection of targets in resource-poor settings. Interestingly, using the integrated paper-based loop-mediated isothermal amplification (LAMP)-LFA, we successfully performed highly sensitive and specific target detection, achieving a detection limit of as low as 3×10^3 copies of target DNA, which is comparable to the conventional tube-based LAMP-LFA in an unintegrated format. The device may serve in conjunction with a simple paper-based sample preparation to create a fully integrated paper-based sample-to-answer diagnostic device for point-of-care testing (POCT) in the near future.

Introduction

Nucleic acid testing (NAT) plays an essential role in medical diagnostics, food safety analysis and environmental monitoring due to its relatively higher sensitivity and specificity than antigen or antibody detection.[1–3] However, the amount of target nucleic acid in a raw sample is usually too low to be directly detected, hence requiring an amplification process. Conventionally, nucleic acid amplification and detection (*e.g.*, polymerase chain reaction (PCR) and electrophoresis) are separately performed, which are costly, labour-intensive, time-consuming and large equipment dependent, reducing their suitability for point-of-care (POC) applications.[4–6]

Nowadays, there exist a number of nucleic acid-based diagnostic equipment available in the market with the ability to perform isothermal amplification (*e.g.*, loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA))[7,8] and/or amplicon detection. For instance, isothermal amplification with real time detection has been implemented in several systems such as the SAMBA system by Diagnostics for the Real World (UK), Loopamp real-time turbidimeter (LA-200) by Eiken (Japan), Twista portable real-time fluorometer by TwistDX (UK) and bioluminescence assays in a real-time (BART) instrument by Lumora (UK). Even though these instruments are simpler, more portable and enable more rapid detection as compared to conventional benchtop NAT equipment, most of them involve fluorescence detection, which requires implementation of a UV light source, thus adding to the entire cost. To this end, simpler colorimetric detection has been exemplified by the BeSt Cassette by BioHelix (USA) and the XCP nucleic acid detection device by Ustar Biotech (China). However, the amplification process requires an electric-heated thermostatic water bath or block heaters,[9,10] and is separately performed from detection, which entails multiple processing steps, hence restricting their use at the POC. Similar drawbacks have also been shown in several studies, which perform tube-based isothermal amplification, followed

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by colorimetric,[11] fluorescence[12,13] or chemiluminescence detection,[14,15] either in real-time[12,13] or endpoint detection.[11,14,16] Therefore, the integration of both nucleic acid amplification and detection into a simpler and more cost-effective platform is in high demand, especially in resource-limited settings.

Paper has gained increasing interest as a promising platform for point-of-care testing (POCT) due to its simplicity, cost-efficiency, biodegradability, and biocompatibility.[17,18] The porous structure of paper enables fluids to wick through *via* capillary action,[19,20] offering advantages such as sequential delivery of reagent,[21,22] sample mixing, and multiple-step processing,[23] which could potentially simplify the existing nucleic acid amplification technique. This emerging technique offers great potential to meet the Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, and Deliverable to end-users (ASSURED) criteria outlined by the World Health Organization (WHO).[24] In recent years, many studies have demonstrated the lateral flow assay (LFA) for amplicon detection due to its simple colorimetric readout, but a complex benchtop nucleic acid amplification process is still required prior to the detection, leading to increased overall assay cost and time.[9,10,25,26] To address these limitations, paper-based isothermal amplification techniques have been introduced. Recent studies have demonstrated the use of paper as a platform for RPA[27] and helicase-dependent amplification (HDA),[28] followed by LFA for the detection of human immunodeficiency virus (HIV) and *Chlamydia trachomatis*, respectively. However, the paper-based amplification and LFA were still separately performed, which entails multiple processing steps, hence increasing the risk of reagent loss and even cross-contaminations. To date, a great challenge remains in integrating paper-based amplification platform into a simple colorimetric LFA, which could tremendously simplify the operation steps. The challenge would be the requirement for on-chip fluidic control from the nucleic acid amplification zone to the lateral flow strip, with optimum temperature required for each step in a robust and portable manner. Developing an integrated LFA without relying on large equipment (*e.g.*, a thermal cycler, an electric heater, an incubator or a water bath) for amplification and an extra UV source for assay readout is of great importance for on-site NAT. However, to the best of our knowledge, no study has attempted to combine paper-based nucleic acid amplification and detection in an integrated lateral flow strip. In addition, a handheld battery-powered heater to be used for nucleic acid amplification is imperative to be coupled with the integrated LFA, which, however, has not yet been introduced for use in resource-poor endemic areas. Therefore, there is a strong demand to develop a new colorimetric integrated LFA that can achieve on-site naked-eye detection.

In this study, considering the simplicity of the device in conjunction with the high efficiency of nucleic acid amplification, we developed an integrated paper-based device incorporating paper-based LAMP and LFA (Fig. 1A). LAMP, a well-known novel nucleic acid amplification technique with high sensitivity and specificity under isothermal conditions, is performed to produce a large number of amplicons, followed by signal detection by LFA and quantification using a smartphone. The paper-based LAMP and lateral flow strip were initially separated by hydrophobic polyvinyl chloride (PVC) layers, creating the "valves" to control the fluid flow from the amplification zone to the test strip. The integrated paper-based device was coupled with a specially designed handheld battery-powered system to enable target detection in remote settings without the need for an external electrical power supply (Fig. 1B). Recently, as dengue infections have become a serious healthcare concern worldwide, including underdeveloped and developing countries, there is an urgent need to develop simple, rapid and robust diagnostic tools for dengue detection in resource-poor settings. Therefore, we used dengue viral DNA as a model analyte in this study. With this integrated paper-based amplification-to-detection device, we successfully achieved a higher assay sensitivity with a detection limit of as low as 3×10^3 copies as compared to an unintegrated platform (3×10^6 copies). Interestingly, there was no significant difference between the detection limit of tube-based LAMP-LFA and integrated paper-based LAMP-LFA. The device permits the potential use of other isothermal amplification techniques, by adjusting the temperature. We are developing a simple paper-based sample preparation technique to be integrated into this paper-based platform to create a fully integrated paper-based sample-to-answer molecular diagnostic device, which is feasible for the detection of a variety of target analytes in POC settings in the near future.

Experimental section

Lateral flow strip fabrication

Gold nanoparticles (AuNPs) with a diameter of 13 ± 3 nm and AuNP–DP conjugates were prepared and all the components of the lateral flow strip were assembled according to the published protocol.[26] Streptavidin was used to immobilize the control probe on the nitrocellulose membrane. The control probe dry powder (15.9 nmol) was added to 121 μL of 2 mg mL^{-1} streptavidin–PBS solution and 22 μL of PBS. About 22 μL ethanol was added to the solution following 1 h incubation at room temperature. About 0.5 μL of 100 μM control probe was then dispensed onto the control zone. As for the test zone, about 0.5 μL of 2 mg mL^{-1} streptavidin was dispensed onto it. After adding 10 μL of AuNP–DNA conjugates to each test strip, they were then dried at 37 °C for 2 h prior to the assay.

Fabrication of an integrated paper-based amplification-to-detection device

An integrated paper-based amplification-to-detection device was specially developed for cost-effective nucleic acid amplification and lateral flow detection at the POC. The two-layer integrated device was fabricated by the combination of the lateral flow strip (first layer) and the LAMP device (second layer), with the fabrication process depicted in Fig. 1A. Briefly, the LAMP device is composed of a piece of glass fiber pad (1 cm ×

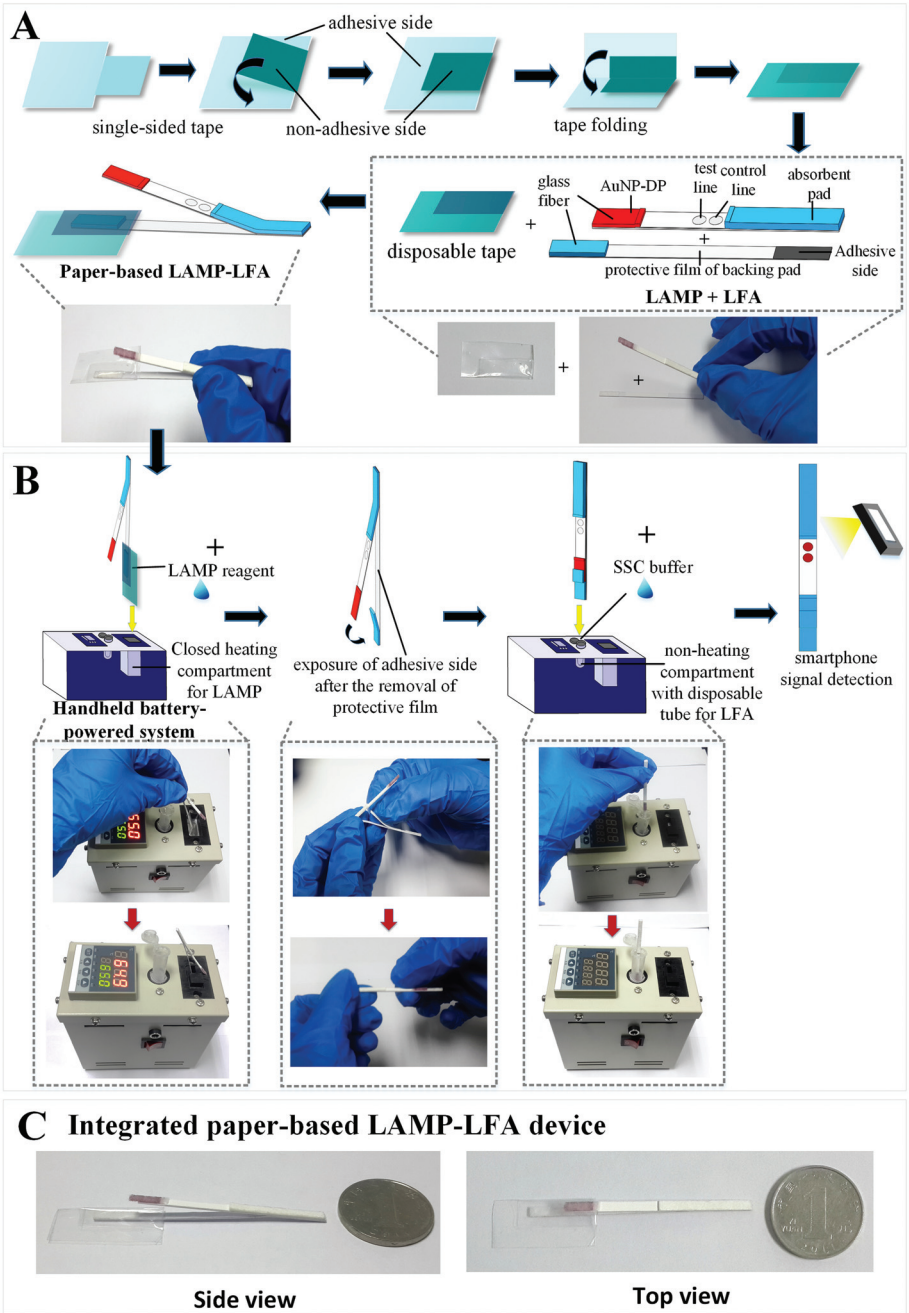

Fig. 1 An integrated paper-based device incorporating LAMP and LFA. (A) The fabrication process of paper-based LAMP-LFA. (B) A handheld battery-powered system was developed for paper-based amplification and detection of nucleic acid, coupled with smartphone signal detection in resource-poor POC settings. (C) The real image of integrated paper-based LAMP-LFA device.

0.25 cm) protected by an adhesive tape and an adhesive PVC backing pad. The protective films at the two ends of the backing pad were first removed to expose the adhesive surface. The glass fiber pad was then mounted onto one end of the backing pad, to create a glass fiber pad–backing pad LAMP device, while another end of the backing pad was attached to the lateral flow layer. A piece of 3.5 cm × 2 cm disposable adhesive tape was folded into half, creating a small pocket that acts as a reaction chamber, into which the glass fiber pad of the LAMP strip is inserted. Both the LAMP device and the lateral flow strip (fabricated as previously described) were combined together to create an integrated amplification-to-detection device. The entire fabrication process required less than 1 min.

LAMP optimization

In NAT, detection of RNA viruses (*e.g.*, dengue viruses) normally involves the process of RNA extraction, reverse-transcription and amplification and finally target DNA detection.[29,30]

In this study, we used synthetic dengue viral DNAs as model analytes for proof-of-principle investigation of the potential of our prototype integrated LFA to perform both isothermal amplification and detection using the handheld battery-powered system. LAMP was performed in a total of 25 μL reaction mixture using a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd, Japan) containing the reaction mix (consisting of 40 mM Tris-HCl, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M betaine, 2.8 mM each of dNTPs), Bst DNA, 40 pmol each of the primers FIP and BIP, 5 pmol each of the outer primers F3 and B3, 20 pmol each of the loop primers F and B and the specific amounts of target DNA. The oligonucleotide primers used in this study were DEN-1 Western Pacific (U88535) serotype specific primers, which were synthesized based on the sequences reported in the literature[31] with specially designed control and detector probes. Briefly, the conserved regions were identified using DNASIS software (Hitachi, Japan) and all the primers were designed using primer design software (Net Laboratory, Japan). All the sequences used were obtained from Sangon Biotechnology Co., Ltd (Shanghai, China) (Table 1). As the target DNA used in this study is typically long (199 bp), the DNA was inserted into a commonly used pUC57 plasmid isolated from *E. coli* strain DH5α. The vector length is about 2710 bp.[32] In order to investigate the effectiveness of paper-based LAMP over a range of temperatures, LAMP was performed in an unintegrated paper (glass fiber pad) placed in the tube at a range of temperatures (58, 60, 63, 65, and 68 °C) for 1 h using a thermal cycler (Veriti, Applied Biosystem, Foster City, USA) and the data were compared with the control group (tube-based LAMP). With the optimum temperature of 63 °C, a series of concentrations of the DNA template (3×10^{10}, 3×10^{8}, 3×10^{6}, 3×10^{4} and 3×10^{2} copies) were tested at a range of incubation times (15, 30, 45, 60 and 90 min).

Endpoint target detection

Following the LAMP, the amplicon was collected by centrifugation of the tube containing the glass fiber pad. The amplicon was then detected by gel electrophoresis with 3 μL of the LAMP products being subjected to 1.5% agarose gel electrophoresis. Fluorescence staining was also performed with SYBR green I stain (Invitrogen, USA). The products were detected visually by a colour change from the original orange to yellowish-green following the addition of 2 μL 1000-fold diluted SYBR Green I dye to the tube to obtain the final concentration of about 1×. The concentration of the amplicon was further confirmed by measuring the absorbance value at 260 nm (OD 260) with the nanodrop ND-2000 (Thermo Scientific).

As for the LFA, a denaturation step was required prior to the LFA by heating the amplicon in the tube at 95 °C with the optimum denaturation period of 0.5 min using a water bath. The amplicon was then mixed with 50 μL of saline sodium citrate (SSC) buffer, and the mixture was then added into the 96-well plate. LFA was performed and the colour formation in the test zone was observed. At the end of the assay, images of all test zones were captured by a smartphone (i-phone 5s), and the colour intensities were converted to optical densities with Image Pro Plus 6.0 software.

Nucleic acid amplification and detection in an integrated paper-based device with the aid of a handheld battery-powered system

The integrated paper-based amplification-to-detection device consists of a glass fiber pad for LAMP, a glass fiber pad for LFA, a nitrocellulose membrane, an absorbent pad and two PVC backing pads (Fig. 1B). The battery-powered system is briefly described in the Results and discussion section. Briefly, the glass fiber pads for LAMP and LFA were initially disconnected. The handheld system was pre-set with the temperature of 63 °C for amplification and 95 °C for amplicon denaturation. The mixture of the samples and LAMP reagents was first pipetted onto the glass fiber pad for LAMP protected by a disposable tape, followed by the amplification process at 63 °C at a range of incubation times (15, 30, 45, 60 and 90 min) in the closed heating compartment of the system. Following the 95 °C denaturation step for an optimum period of 0.5 min (30 s), both glass fiber pads were then connected through the removal of the protective film of the PVC backing pad. The integrated device was moved to the non-heating compartment composed of a disposable microcentrifuge tube to perform LFA following the addition of 80 μL SSC buffer to the tube. To determine the detection limit of the assay, a sensi-

Table 1 Oligonucleotide sequences used for LAMP

Name	Genome position	Sequence (5′–3′)
Detector probe	10595–10610	5′-tagaggttagaggaga-$(CH_2)_6$-SH-3′
Control probe	10595–10610	5′-tctcctctaacctcta/biotin-3′
F3	10469–10487	5′-gaggctgcaaaccatggaa-3′
B3	10648–10667	5′-cagcaggatctctggtctct-3′
FIP	10489–10506/TTTT/10529-10549	5′-Biotin/gctgcgttgtgtcttgggaggttttctgtacgcatggggtagc-3′
BIP	10557–10576/TTTT/10612-10629	5′-cccaacaccaggggaagctgtttttttgttgttgtgcggggg-3′
FLP	10508–10525	5′-ctcctctaaccactagtc-3′
BLP	10578–10593	5′-accctggtggtaagga-3′
DENV target DNA	10469–10667	5′-gaggctgcaaaccatggaagctgtacgcatggggtagcagactagtggtt agaggagacccctcccaagacacaacgcagcagcggggcccaacaccagggg aagctgtaccctggtggtaaggactagaggttagaggagacccccgcacaacaac aaacagcatattgacgctgggagagaccagagatcctgctg-3′

tivity assay was performed with a series of dilutions of the amplicon (3×10^{10}, 3×10^{8}, 3×10^{6}, 3×10^{4}, 3×10^{3} and 3×10^{2} copies) using the integrated paper-based device. The data were compared with those obtained using the tube-based LAMP, followed by LFA. The colour intensities of test zones were observed and their optical densities were determined.

Statistical analysis

Statistical analysis was performed using one-way ANOVA with the Tukey *post-hoc* test to compare the data among different groups in LFA optimization assays. Data were expressed as the mean ± standard error of the mean (SEM) of three independent experiments ($n = 3$). $p < 0.05$ was reported as statistically significant.

Results and discussion

In fact, in nucleic acid testing (NAT), RNA viruses such as dengue virus,[33] Human Immunodeficiency Virus (HIV)[34–36] and Hepatitis C Virus (HCV)[34,37] normally require reverse transcription and amplification prior to target detection. However, DNAs have been commonly used for evaluation of the performance of prototypes for various medical diagnostics.[27,38,39] In this study, we focused on the proof-of-principle investigation of the potential of our prototype integrated LFA to perform both amplification and detection using the handheld battery-powered system. We used synthetic dengue viral DNAs as the model analyte and we suggest that this technique can be broadly applied to other target DNA/RNA.

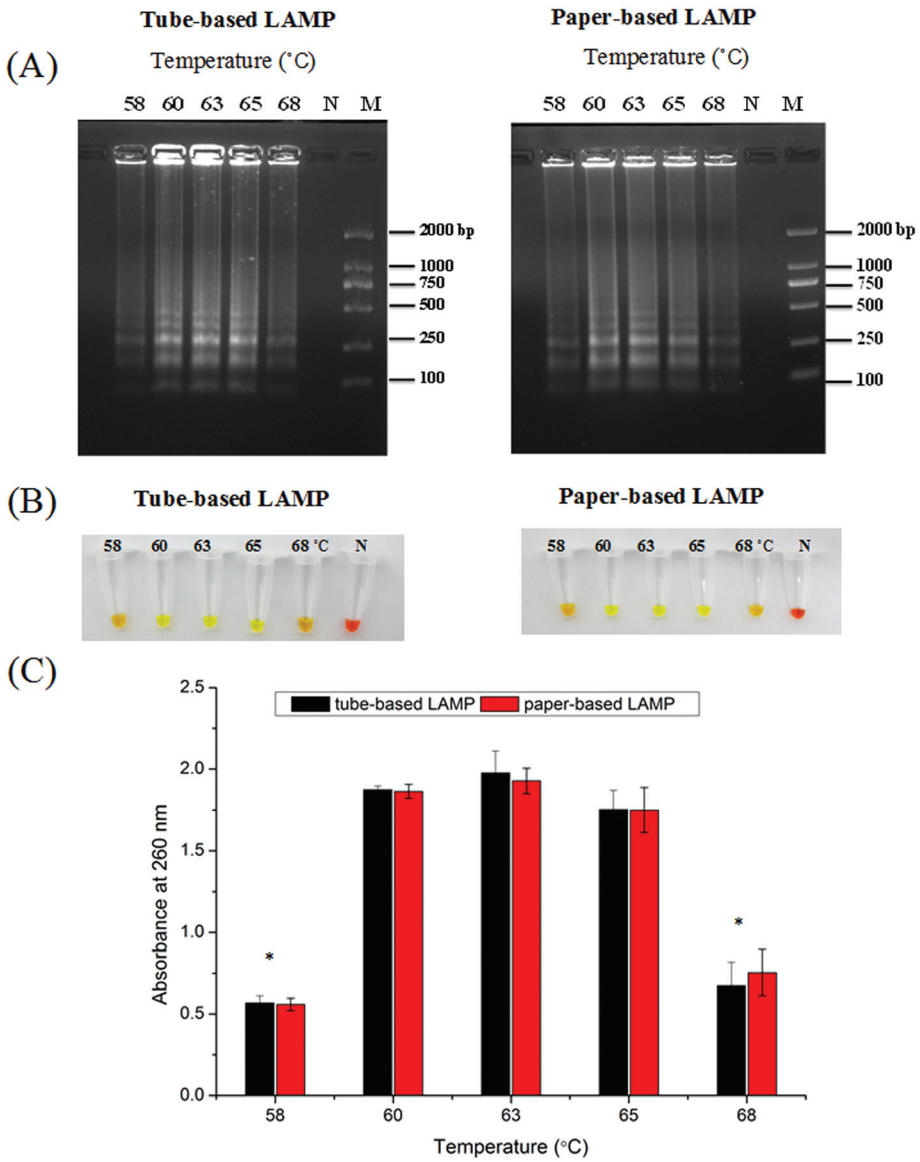

Fig. 2 Optimization of the LAMP temperature. The LAMP temperature of 63 °C, as the middle point, was the optimum LAMP temperature based on the visible bands in electrophoresis for both tube-based and paper-based platforms (A), the comparable positive result shown (yellowish-green) after SYBR Green I staining (B) and the absorbance value at 260 nm (* $p < 0.05$) (C) (N = negative control, M = 100–2000 bp marker).

Initially, to optimize the LAMP reaction temperature, the amplification process was performed at a range of temperatures (58, 60, 63, 65 and 68 °C) for 60 min in a conventional tube-based and paper-based platform, a glass fiber pad, which has been reported as a suitable matrix for amplification,[27] using the handheld heating system. Similar to the tube-based LAMP, the optimum temperature for the paper-based LAMP was 63 °C, as indicated by more clearly visible bands shown in electrophoresis (Fig. 2A), a denser yellowish-green colour produced in SYBR Green I staining (Fig. 2B) and a higher absorbance value obtained at 260 nm (OD 260) (Fig. 2C). Our data support the notion that 60–65 °C is the optimum temperature for the Bst DNA polymerase activity.[40,41]

To demonstrate the ability of a paper-based platform to effectively support the amplification, at the optimum temperature of 63 °C, LAMP was performed in the glass fiber pad (*i.e.*, "unintegrated" paper-based LAMP) with a series of concentrations of the DNA template (3×10^{10}, 3×10^{8}, 3×10^{6}, 3×10^{4}, 3×10^{3} and 3×10^{2} copies) over a range of incubation times (15, 30, 45, 60 and 90 min) using the handheld heating system. The glass fiber pad was protected by the disposable tape using the handheld heating system. The negative result shown in the negative control suggests that the potential contamination has no significant effect. The result of agarose gel electrophoresis shows that the longer the incubation time, the higher the amount of amplicons produced. The brightness of the bands appears to be proportional to the amount of DNA template used, as indicated by the detection limit of as low as 3×10^{6} copies achieved at the incubation time of at least 60 min (Fig. 3A). These data were further supported by SYBR Green I staining (Fig. 3B).

As for the lateral flow-based endpoint detection, a schematic diagram of the process of amplification to detection is depicted in Fig. S1.† As DNA–DNA binding possesses a substantially higher specificity than antigen–antibody interaction, instead of using protein (*i.e.* antibody), we used a DNA probe (AuNP–DP) to hybridize with the target DNA. Briefly, following the amplification process, a denaturation step is required to

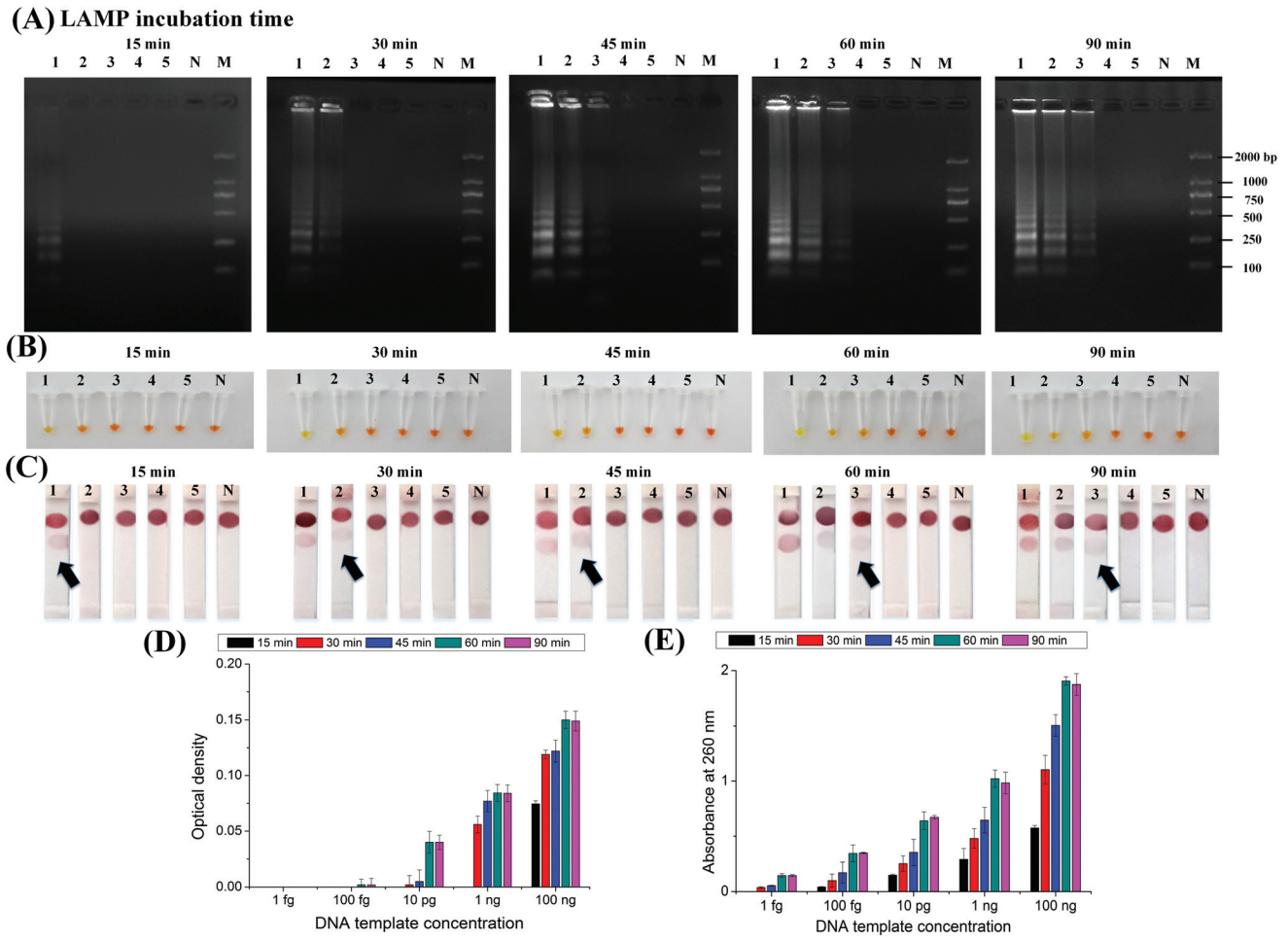

Fig. 3 Unintegrated paper-based LAMP and LFA. With the optimum LAMP temperature (63 °C), LAMP performed with a series of concentrations of the DNA template in the paper-based platform at different incubation times. The data of gel electrophoresis showed a directly proportional relationship between the brightness of the bands and the concentration of DNA template for LAMP (A). The positive result was further supported by the yellowish-green colour solution observed after the SYBR Green I staining (B), LFA (C & D) and the absorbance value at 260 nm (E) (1 = 3×10^{10} copies, 2 = 3×10^{8} copies, 3 = 3×10^{6} copies, 4 = 3×10^{4} copies, 5 = 3×10^{2} copies, N = negative control, *M* = 100–2000 bp marker).

separate the double-stranded DNAs into single strands at a denaturation temperature of 95 °C at an optimum period of 0.5 min (Fig. S2A†). The single-stranded target would then bind to the single-stranded detector probe (DP) coupled with gold nanoparticles (AuNPs) (AuNP–DP). After diffusing across the nitrocellulose membrane, the biotinylated amplicons would be captured by the streptavidin at the test zone to produce a red signal, with an optimum streptavidin concentration of 2 mg mL^{-1} (Fig. S2B†). The remaining AuNP–DP would then hybridize with the control probe to form a red signal. In line with the results of electrophoresis and fluorescence staining, LFA showed a significantly higher optical density in the sample with a higher quantity of DNA template and a longer incubation time. A detection limit in LFA could be achieved of as low as 3×10^6 copies at the optimum incubation time of 60 min (Fig. 3C and D). The amplification efficiency was further confirmed by the measurement of the absorbance value (OD 260) of the amplicon (Fig. 3E). Collectively, 60 min was indicated as the optimum incubation time to achieve the lowest detection limit of the assay.

In fact, a suitable probe design could be used to eliminate the step of denaturation to simplify the entire DNA amplification-to-detection process. Furthermore, utilizing a relatively longer spacer between the detector probe and its thiol group could reduce the potential steric hindrance in the hybridization between the detector probe and the target analyte, which may improve the performance of LFAs. In this study, however, our main purpose is to prove the concept of an integrated LFA that can perform both DNA amplification and detection. All the optimizations in the probe design will be investigated in our future study.

To simplify the processing step and achieve maximum sensitivity of the assay, integrating paper-based LAMP into LFA is proposed, in order to create an integrated paper-based LAMP-to-LFA device for optimum amplicon detection. In fact, using unintegrated paper-based LAMP and LFA initially, we found that there was a loss of more than half the amount of solution from the original volume of 25 μL, with the average solution volume of 9.67 ± 0.33 μL left in the tube throughout the process. This might be due to the need for an additional step of centrifugation for amplicon collection between the process of paper-based LAMP and LFA, which could be successfully solved using the integrated device. The challenge in combining paper-based LAMP and LFA would be the requirement for on-chip fluidic control from the LAMP zone and the lateral flow strip with a different temperature and time required for each zone. We addressed the challenge by creating the "valves" made of hydrophobic polyvinyl chloride (PVC) to control the fluid flow from one zone to another through connecting the PVC layers. The top PVC layer supports the lateral flow strip, which consists of a glass fiber pad, a nitrocellulose membrane and an absorbent pad. The bottom PVC layer is comprised of a glass fiber pad, which acts as a platform for LAMP.

A piece of disposable adhesive tape was folded into half, creating a small pocket to cover the amplification zone (glass fiber pad) to prevent sample evaporation and contamination as previously described. To investigate the potential risk of sample evaporation in the integrated biosensor, the mass of the device was recorded before and after heating at 63 °C. By using a tape as a protector, the absence of sample evaporation was observed, as indicated by no significant difference in the mass of the biosensor before and after the amplification (Fig. S3†). There was also no risk of contamination throughout the process, as evidenced by the negative result shown by all the negative controls.

As high temperature is normally required for nucleic acid amplification, developing a handheld battery-powered system is essential to couple it with the integrated paper-based device for target detection in resource-limited settings where electricity may not be available.[42] Therefore, a handheld battery-powered system was specifically designed for performing nucleic acid amplification and detection in an integrated paper-based device (Fig. 4A). This handheld system consists of a closed heating compartment for amplification, a non-heating testing compartment for target analyte detection, an integrated battery, an integrated temperature controller and a charger. The heating compartment is made internally of aluminium alloy with an external insulation wall, whereas the testing compartment is made of chemical-resistant polyformaldehyde, which could fit well with various sizes of disposable microcentrifuge tubes for target analyte detection. A battery is integrated into the system with a programmable temperature controller for temperature control with a range from room temperature +5 °C to 100 °C, with the resolution of ±0.1 °C. The temperature sensor is installed in the internal part of the system, with the temperature displayed at the exterior part to ensure the maintenance of the optimum temperature for the process. The heating process is required only for amplification; therefore the system could be turned off for LFA for power saving. Both nucleic acid amplification and detection could also be simultaneously performed with this handheld system. The specifications of the device are summarized in ESI Table 1†. This portable and cost-effective system, coupled with a simple paper-based amplification-to-detection device, enables diagnosis of infectious diseases in POC settings.

To investigate the potential of this simple and portable testing system to facilitate the integrated paper-based amplification-to-detection device in remote settings, test assays were performed using this system. Following the amplification at 63 °C and denaturation at 95 °C in the closed heating compartment, both the amplification zone and the lateral flow strip were then connected. LFA was then performed in the non-heating compartment, followed by signal detection by a smartphone. Interestingly, the optimum amplification period of 60 min was found to be capable of successfully achieving a detection limit of as low as 3×10^3 copies for dengue viral DNA (Fig. 4B and C), which was more sensitive than that of unintegrated paper-based LAMP and LFA (3×10^6 copies) due to the ability to retain the optimum amount of amplicons for detection in a single device. There was no significant difference between the detection limit of tube-based LAMP-LFA and integrated paper-based LAMP-LFA (Fig. 4B and C), highlighting

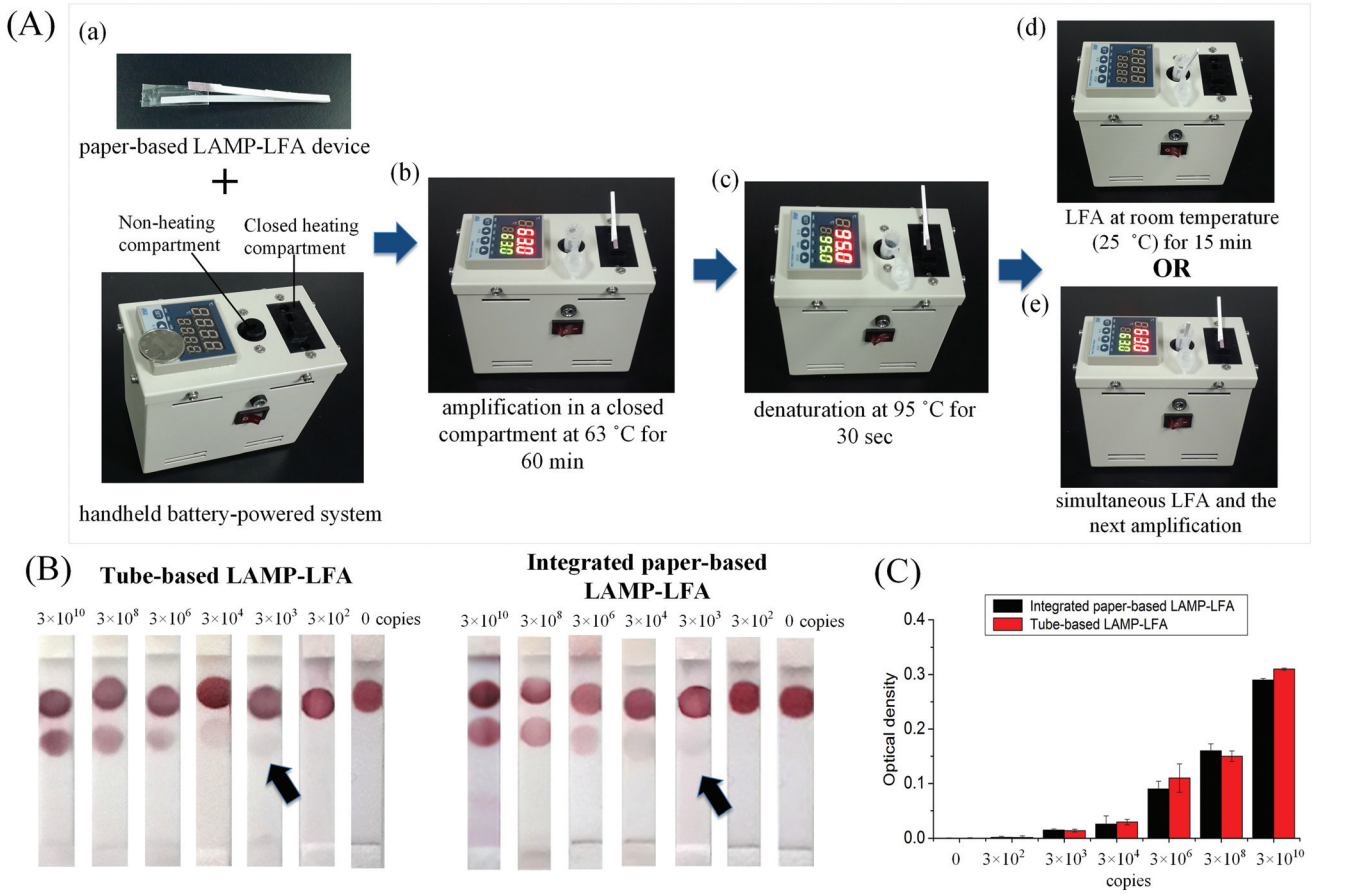

Fig. 4 An integrated paper-based LAMP-LFA device coupled with a handheld testing system. (A) Photographic images of the paper-based LAMP-LFA device to be combined with a handheld system (a) for LAMP in a closed heating compartment (b), followed by nucleic acid denaturation (c) and LFA in a non-heating compartment. The LFA could be performed without power supply (d) or simultaneously performed with the next LAMP (e). (B & C) The integrated paper-based device could achieve the detection limit of as low as 3×10^3 copies at the optimum incubation time of 60 min, which was comparable with that of tube-based LAMP-LFA in an unintegrated format.

its potential use for sensitive target detection in the near future. The sensitivity of the integrated device was comparable to or even higher than that of the existing studies.[43,44] Even though some studies have reported more sensitive target detection (~100 copies),[31,45] the amplification and detection were separately performed. Besides that, different targets and probes may yield different detection sensitivities. In the future, our group will fully optimize the probes and perform clinical sample testing using our prototype to prove highly sensitive target detection. Additionally, we suggest further improving the sensitivity of LFA using a simple sensitivity enhancement method in LFA (*e.g.*, paper architecture modifications[46] or integration of wax-printed pillars[47]).

Conclusion

In short, we developed an integrated paper-based device to perform both nucleic acid amplification and detection with the aid of a handheld system, which promises sensitive and specific target detection. The capability of producing colorimetric signal detectable by the naked eye eliminates the requirement of a UV source for assay readout. The utilization of a specially designed system eradicates the need for high-end equipment commonly used for amplification such as a thermal cycler, an electrical heater or a water bath, suggesting its potential use in POC settings. Given that this handheld system is regulated by a programmable temperature controller with the temperature ranging from 25 to 100 °C, it is suggested that other amplification techniques could also be applied. The technique described here could also be used to detect a variety of target nucleic acids. The integration of a simple paper-based extraction method into this device could facilitate sample-in-answer-out NAT in remote settings.

However, as the present study represents a proof-of-principle study, a number of limitations have to be addressed in potential real world applications. To achieve contamination-free fully integrated NAT, future work should focus on developing a portable closed system for the entire sample-in-answer-out process as reported in the literature.[48,49] To eliminate the

requirement for a laboratory unit (*e.g.*, a refrigerator) for reagent storage and multiple processing steps (*e.g.*, pipetting) which would increase the risk of contamination, future work should include on-chip reagent storage (*e.g.*, hydrogel as a fluid reservoir[50] or on-chip dry reagent storage[51]). Additionally, to increase the detection sensitivity, future work should focus on integrating a simple fluidic control strategy into LFA (*e.g.*, paper architecture modifications[46] or integration of wax-printed pillars[47]) with simple fabrication and operation steps. To address the tedious operation steps, incorporating paper-based valve technology into the paper-based device[52] is also essential to enable automated sequential fluid delivery. Furthermore, a suitable probe design should be used to eliminate the step of denaturation, to simplify the entire DNA amplification-to-detection process. We envision that our next generation of integrated systems will be simple, portable, disposable, cost-effective, user-friendly and highly sensitive with an automated sample-to-answer capability to achieve rapid NAT at the POC.

Conflict of interest

All authors declare no conflict of interest.

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