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An integrated paper-based sample-to-answer biosensor for nucleic acid testing at the point of care†

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With advances in point-of-care testing (POCT), lateral flow assays (LFAs) have been explored for nucleic acid detection. However, biological samples generally contain complex compositions and low amounts of target nucleic acids, and currently require laborious off-chip nucleic acid extraction and amplification processes (*e.g.*, tube-based extraction and polymerase chain reaction (PCR)) prior to detection. To the best of our knowledge, even though the integration of DNA extraction and amplification into a paper-based biosensor has been reported, a combination of LFA with the aforementioned steps for simple colorimetric readout has not yet been demonstrated. Here, we demonstrate for the first time an integrated paper-based biosensor incorporating nucleic acid extraction, amplification and visual detection or quantification using a smartphone. A handheld battery-powered heating device was specially developed for nucleic acid amplification in POC settings, which is coupled with this simple assay for rapid target detection. The biosensor can successfully detect *Escherichia coli* (as a model analyte) in spiked drinking water, milk, blood, and spinach with a detection limit of as low as 10–1000 CFU mL^{-1}, and *Streptococcus pneumonia* in clinical blood samples, highlighting its potential use in medical diagnostics, food safety analysis and environmental monitoring. As compared to the lengthy conventional assay, which requires more than 5 hours for the entire sample-to-answer process, it takes about 1 hour for our integrated biosensor. The integrated biosensor holds great potential for detection of various target analytes for wide applications in the near future.

Introduction

Molecular diagnostics are currently critical for applications in medical diagnostics, food safety analysis and environmental monitoring.[1–3] Nucleic acid testing (NAT), a molecular diagnostic technique that involves nucleic acid extraction, amplification and detection, conventionally relies on well-established laboratories, high-end instrumentation and highly trained operators, limiting its use in resource-poor settings.[4–6] With the increasing incidence of infectious diseases and food and water contamination, particularly in developing and underdeveloped countries with poor infrastructure,[7,8] there is an urgent need to develop simple, inexpensive, portable and rapid molecular diagnostic tools, which can be readily implemented in remote settings.[9] Recent advances in point-of-care (POC) testing, especially lateral flow assays (LFAs), make it possible to achieve simple and cost-effective NAT at the POC.[10–12] LFAs are able to produce results in a simple way (visible colour formation) in less than 30 min. However, as biological samples (*e.g.*, blood, urine, saliva) are generally complex and contain low amounts of target nucleic acids, substantial off-chip

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extraction and amplification processes (*e.g.*, tube-based extraction and polymerase chain reaction (PCR)) are normally required prior to lateral flow detection.[13–15] Recently, paper-based extraction and amplification has been introduced for detection of various diseases (*e.g.*, HIV,[16] *Chlamydia trachomatis*[17] and influenza A[18]). However, these essential steps have been separately performed from LFAs, which entail multiple processing steps, hence increasing the risk of reagent loss and cross-contamination. Therefore, it is of great importance to integrate nucleic acid extraction, amplification and lateral flow detection in an integrated paper-based biosensor for use in remote settings.

Although there has been a growing interest in developing low-cost integrated sample-to-answer biosensors for POC applications,[19] there are only a few studies which report on this. For instance, Whitesides' group has developed an integrated "paper machine" by incorporating sample preparation, nucleic acid amplification and signal detection.[20] An electrically-powered heater and an external UV source were used for amplification and fluorescence signal detection, respectively. To date, a great challenge remains in integrating LFA into one single biosensor, which could tremendously simplify the final readout. The challenge would be the requirement for on-chip fluidic control from the nucleic acid extraction zone to the amplification zone and lateral flow strip, with optimum temperature required for each NAT step in a robust and portable manner. To the best of our knowledge, even though the integration of DNA extraction and amplification into a paper-based biosensor has been reported, a combination of LFA with the aforementioned steps for simple colorimetric readout has not yet been demonstrated. In addition, a handheld battery-powered heater to be used for nucleic acid amplification is imperative to be coupled with the integrated biosensor, which, however, has not yet been introduced for use in low-resource endemic areas. Therefore, there is a strong demand for a new colorimetric integrated paper-based molecular biosensor that can achieve rapid on-site naked-eye detection.

In the present study, we developed a prototype paper-based biosensor, where a Fast Technology Analysis (FTA) card and glass fiber were integrated into a lateral flow strip for nucleic acid extraction and amplification, followed by naked eye detection and quantification using a smartphone. All paper matrices were initially separated by hydrophobic polyvinyl chloride (PVC) layers, creating the "valves" to control the fluid flow from the nucleic acid extraction zone to the amplification zone and lateral flow strip. The integrated biosensor was coupled with a specially designed handheld battery-powered heating device to support highly sensitive and specific loop-mediated isothermal amplification (LAMP), eliminating the need for an electrically-powered heater or thermal cycler. By using *Escherichia coli* (*E. coli*) as the target analyte, we successfully proved that our integrated biosensor could effectively detect a real sample from phosphate buffered saline (PBS), drinking water, milk, blood, and spinach in a simple manner, with a detection limit of 10, 10, 10, 100, 1000 CFU mL^{-1}, respectively, demonstrating its ability in medical diagnostics, environmental monitoring and food safety analysis. We further proved that our biosensor was able to detect the target analyte (*Streptococcus pneumonia*) from a clinical blood sample, indicating its potential clinical application for the future. Instead of requiring more than 5 h in a conventional assay, the entire integrated sample-to-answer process only takes about 1 h. The biosensor permits the potential use of other amplification techniques (*e.g.*, strand displacement amplification (SDA) or recombinase polymerase amplification (RPA)) in remote settings, through adjusting the temperature of the handheld device. This integrated biosensor could be broadly applied to other target analytes at the POC, holding great potential for a wide range of applications.

Materials and methods

Bacteria culture

E. coli ATCC 25922 was used as a model organism in this study. Initially, the bacteria were streaked onto Luria–Bertani (LB) agar containing 100 mg mL^{-1} ampicillin and incubated at 37 °C for 16 hours to allow bacterial growth. An isolated *E. coli* colony was picked and cultured in 10 mL of LB medium at 37 °C for 16 h with agitation at 150 rpm. The *E. coli* culture was used as a standard stock for all experiments. The turbidity of the bacteria suspension was measured at a wavelength of 600 nm (OD 600). The bacteria stock was then diluted ten-fold in PBS and spread on LB-ampicillin plates. The single colonies were then counted after the overnight incubation at 37 °C. Bacteria concentrations were determined by plate colony counting as colony forming units per mL (CFU mL^{-1}), which were further confirmed with OD 600.

Synthesis of gold nanoparticles (AuNPs) and AuNP–DP (detector probes) conjugates

Gold nanoparticles (AuNPs) with diameters of 13 ± 3 nm were prepared according to a previously published protocol.[21] Briefly, in a 250 mL round-bottom flask, 4.5 mL of 1% trisodium citrate and 1.2 mL of 0.825% chloroauric acid were added to 100 mL of boiled distilled water. The colour of the solution changed from yellow to purple and finally turned wine-red in 2 min. The solution was used to prepare AuNP–DP conjugates. Both AuNPs and AuNP–DNA conjugates were characterized by ultraviolet–visible (UV/Vis) spectrophotometry and transmission electron microscopy (TEM, JEM-2100 F).

A thiolated oligonucleotide (detector probe, DP) was used to conjugate with AuNPs. The oligonucleotide was activated before the conjugation, by mixing with 20 μL of 500 mM acetate buffer, 4 μL of 10 nM tris(2-carboxyethyl)phosphine (TCEP) and 100 μL of distilled water. After 24 h, 1% sodium dodecyl sulfate (SDS) and 2 M NaCl were added to the solution to reach a final concentration of 0.01% SDS and 0.16 M NaCl, respectively. Following the centrifugation at 10 000 rpm, the pellet was suspended in 1 mL of eluent buffer, consisting of 5% BSA, 0.25% Tween 20, 10% sucrose and 20

nM Na_3PO_4. The AuNP–DP conjugates have been previously characterized by visible colour changes from wine red to dark red, aggregate formation in transmission electron microscopy (TEM) images, and slight shift of the absorbance values (6 nm) in ultraviolet–visible (UV/Vis) spectra.[21]

Fabrication of an integrated paper-based sample-to-answer biosensor

The integrated paper-based biosensor consists of 4 layers (Fig. 1A). The top PVC layer is the lateral flow layer supported by a PVC backing pad (6 cm × 0.25 cm) (J-B6, Jiening, China), which consists of a glass fiber (1 cm × 0.25 cm) (Pall 8964, Saint Germain-en-Laye, France), a nitrocellulose membrane (1.9 cm × 0.25 cm) (HFB 18002, Millipore, USA), and an absorbent pad (2.5 × 0.25 cm) (H-1, Jiening, China). The second layer is composed of a glass fiber for a highly specific and sensitive nucleic acid amplification technique (*i.e.*, LAMP). The third layer consists of a piece of FTA card (Whatman, UK), with a diameter of 0.25 cm, for sample addition and nucleic acid extraction. The bottom layer is composed of an absorbent pad for sample purification and washing. The waste produced was absorbed by the absorbent pad, which was then removed after the washing process.

By using Matrix™ 2360 Programmable Shear (Kinematic Automation Inc., CA, USA), the assembled pads were cut into small strips with 2.5 mm width. All materials were assembled to create an integrated paper-based sample-to-answer biosensor as depicted in Fig. 1. A piece of 3.5 cm × 2 cm adhesive tape was folded in half, creating a small pocket to cover the amplification zone (glass fiber and FTA card) to avoid evaporation. About 0.5 μL of 2 mg mL^{-1} streptavidin (Promega, USA) and 0.5 μL of 100 μM control probe were dispensed onto the nitrocellulose membrane to create the test zone and the control zone, respectively, on the lateral flow strip.

Optimization of FTA card paper-based extraction in the integrated biosensor

The biological sample was first pipetted onto the FTA card and kept at room temperature for nucleic acid extraction. To achieve rapid nucleic acid extraction for POCT, a range of drying periods (*i.e.*, the cell lysis period) for the FTA card were tested, *e.g.*, 5, 10, 15, 30, 45 and 60 min. Quantitative

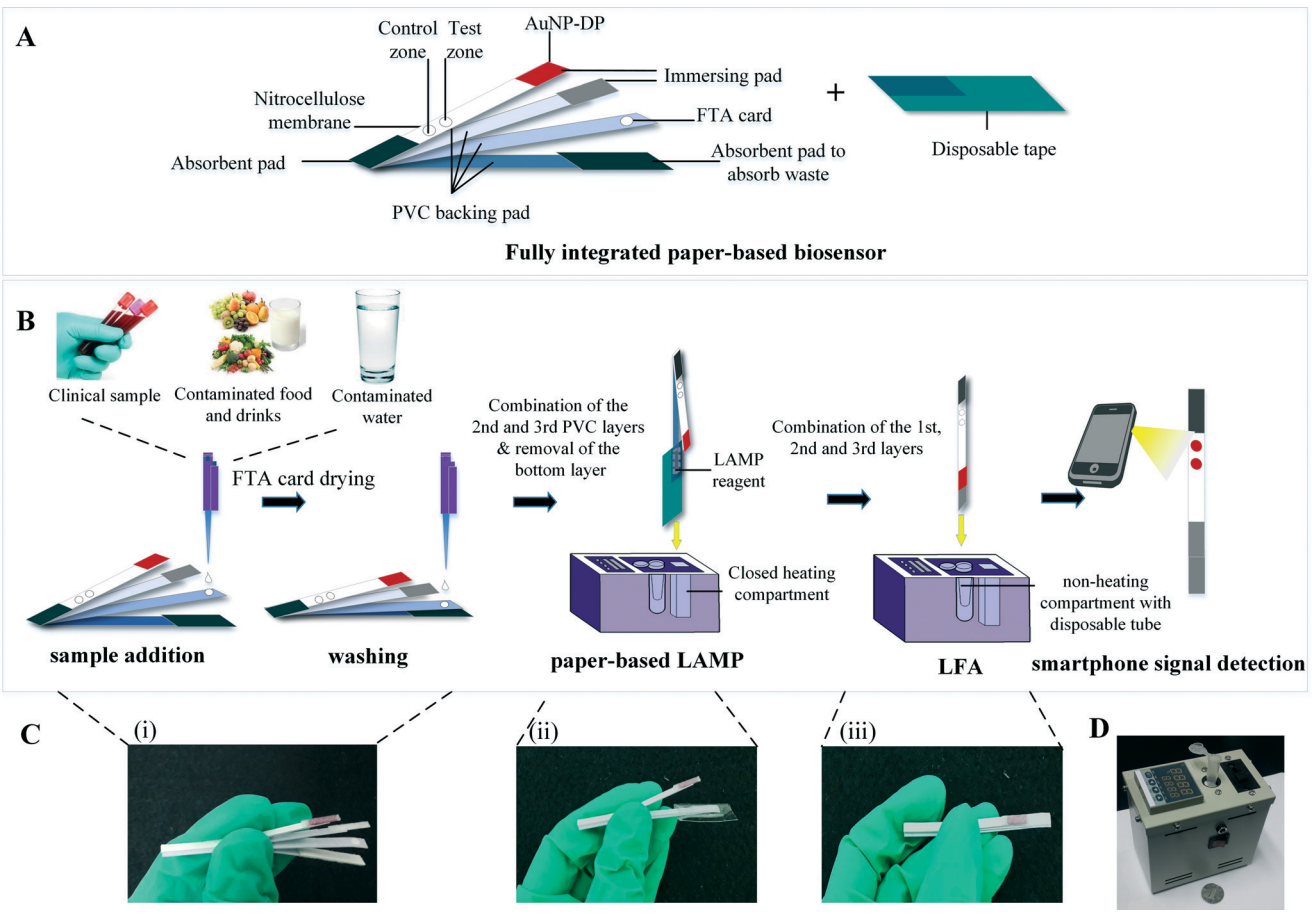

Fig. 1 Schematic of an integrated paper-based sample-to-answer biosensor. (A) The integrated paper-based biosensor is composed of four hydrophobic polyvinyl chloride (PVC) layers to control the sample flow from the nucleic acid extraction zone to the amplification zone and lateral flow strip. (B) The schematic diagram of the experimental procedure. (C) The photo images of the biosensor during the steps of (i) extraction, (ii) amplification and (iii) lateral flow detection. (D) The photo images of a handheld heating device.

Real-Time PCR (qPCR) and LFA were performed to determine the extraction efficiency.

Following drying of the FTA card, a FTA purification reagent (Whatman, UK) and a TE buffer (Sigma-Aldrich) were used to fully wash away the polymerase inhibitors prior to amplification. To determine the volume of the FTA purification reagent and TE buffer used and the number of washes required, we optimized both factors. Following the optimum 15 min of drying for the FTA card, we tested our biosensor with various volumes of the FTA purification reagent and TE buffer (*e.g.*, 20 μL and 40 μL, 40 μL and 80 μL, and 80 μL and 160 μL respectively). We also investigated the necessity of one, two or three washes to fully remove the polymerase inhibitors.

Optimization of paper-based LAMP temperature and reaction period

All sequences used in the study were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China) (Table S1†). Loop primers were used to accelerate the reaction by providing more starting sites for the LAMP cycling process.

Following nucleic acid extraction, the second and third layers of PVC were combined, followed by the addition of amplification reagents onto the tape-covered glass fiber for amplification. The biosensor was then moved into the covered heating compartment of the handheld heating device for the amplification process. To investigate the paper-based LAMP temperature, the amplification was performed over a range of temperatures (58, 60, 63, 65 and 68 °C) for 60 min, followed by detection by electrophoresis and SYBR Green I staining. The LAMP products were observed under visible light and UV light.

LFA in the integrated biosensor

Following the amplification, a denaturation step at 95 °C for 30 s is required to separate the double-stranded DNAs into single strands to be hybridized with AuNP–DP. The glass fiber from the second layer was combined with the top lateral flow layer, and was moved into the non-heating compartment containing the disposable microcentrifuge tube to perform LFA upon the addition of 50 μL of SSC buffer. At the end of each assay, images of all test zones were captured with a smartphone, and the colour intensities were converted to optical density with an App developed by our group. The data were then statistically analyzed to determine the detection limit.

Various biological sample tests

The bacteria were first diluted in PBS for optimization of paper-based extraction and LAMP. To further show the potential of the biosensor to be applied in medical diagnostic, environmental monitoring and food safety analysis, we spiked the bacteria into drinking water, milk, blood, and spinach samples with the final concentrations ranging from 1 to 10^5 CFU mL^{-1}. Discarded whole blood was used in this study. Bottled water, milk (containing 3% energy, 5% protein, 6% fat, 2% carbohydrate, 3% sodium and 13% calcium) and spinach samples were obtained from a local grocery store. Spinach leaves were washed and were then spiked with *E. coli* before mixing with 100 mL of ultrapure water in a blender. The mixture was then filtered using a 70 μM-cell strainer to remove the residues of the leaves before testing. The mixture of each biological sample and *E. coli* was vortexed for 30 s prior to NAT.

Clinical blood sample testing

To prove the ability of the biosensor to detect other types of bacteria, *Streptococcus pneumonia* in clinical blood samples was tested. Having the *S. pneumonia* probes and primers, the biosensor is called *S. pneumonia* biosensor. With prior informed written consent, human blood samples from six patients with pneumococcal bacteremia and two healthy donors were obtained from the First Affiliated Hospital of Xi'an Jiaotong University. The study was approved by the Institute Research Ethics Committee of The First Affiliated Hospital. Six *S. pneumonia*-positive samples and two *S. pneumonia*-negative samples confirmed by a gold standard culture method were first tested with conventional benchtop DNA testing, involving tube-based extraction using Purelink Genomic DNA Mini Kits (Invitrogen), tube-based LAMP (components as mentioned) and electrophoresis. The sequences used were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China) (Table S1†). All clinical samples were then tested with the integrated *S. pneumonia* biosensor. To confirm the specificity of the assay using the biosensor, one *S. pneumonia*-positive sample, two clinically confirmed HBV-positive samples and an *E. coli* spiked blood sample were also tested with the *S. pneumonia* biosensor.

Statistical analysis

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

Results and discussion

NAT, which involves extraction, amplification and detection, normally requires high-cost instrumentation, time-consuming processing and skilled personnel, limiting its use in resource-poor settings. Considering the potential of NAT in remote settings for POCT, we developed an integrated paper-based biosensor with a simple colorimetric readout by using gold nanoparticles as an indicator (Fig. 1). The challenge would be the requirement for on-chip fluidic control from the nucleic acid extraction zone to the amplification zone and lateral flow strip with different temperatures and times required for each zone. The presented biosensor, which consists of "valves" made of

hydrophobic polyvinyl chloride (PVC) layers (Fig. 1A), could address the challenge by controlling the fluid flow from one zone to another through connecting the layers (Fig. 1B and C).

The top PVC layer supports the lateral flow strip, which consists of a glass fiber, a nitrocellulose membrane and an absorbent pad. The second layer is composed of a glass fiber, which acts as a platform for highly specific and sensitive LAMP. The third PVC layer consists of a piece of FTA card for sample addition and nucleic acid extraction, and the bottom layer is composed of an absorbent pad which absorbs the waste produced from sample purification and washing. A piece of 3.5 cm × 2 cm adhesive tape was folded into half, creating a small pocket to cover the amplification zone (glass fiber and FTA card) to prevent sample evaporation (Fig. 1B and C). As DNA is amplified in the liquid form, evaporation would affect the efficiency of amplification. To investigate the potential risk of evaporation in the integrated biosensor, the mass of the biosensor was recorded before and after heating at 65 °C. By using a tape as a protector, we found no significance difference in the mass of the biosensor before and after the amplification (Fig. S1†). There was also no risk of contamination throughout the process, as evidenced by the negative result shown by all the negative controls. All abovementioned low-cost materials were assembled to create an integrated paper-based sample-to-answer biosensor as depicted in Fig. 1.

As high temperature is normally required for nucleic acid amplification, developing a handheld battery-powered heating device is essential for coupling with the integrated biosensor for rapid NAT in remote settings. Our device is composed of a heating compartment for amplification and a testing compartment to place a microcentrifuge tube for LFA (Fig. 1B & D). Temperature control in the heating compartment can be achieved by direct current produced by a rechargeable battery-powered source. A battery power supply unit is integrated into the heating device with a programmable temperature controller for temperature control within the range of 25–100 °C with an accuracy of ± 0.1 °C. The battery can last up to 10 h on a single charge. There is a temperature display at the external part of the device to ensure the maintenance of the desired temperature throughout the assay. The specifications of the handheld heating device are summarized in Table S2.†

The whole process is shown in Video S1.† Following the addition of the sample onto the FTA card, the card was allowed to dry and the impregnated chemicals lysed cells at room temperature. As these chemicals may interfere with the downstream analysis, washing steps are required for chemical removal. The bottom layer responsible for waste absorption was removed, and the second and third PVC layers were combined, followed by the addition of amplification reagents onto the tape-covered glass fiber for amplification. The tape-covered zone was then moved into the covered heating compartment of the handheld device for amplification. Following the amplification, denaturation was performed to separate the double-stranded DNAs into single strands to be hybridized with AuNP–DP. The second and third layers were then combined with the top lateral flow layer, and were moved into the non-heating compartment containing the disposable microcentrifuge tube for LFA. At the end of each assay, the result could be detected by the naked eye or/and quantified by using a smartphone. The simple integrated paper-based biosensor coupled with this handheld device enables rapid and accurate detection of targets at the POC.

The FTA card was selected for nucleic acid extraction due to its ability to extract nucleic acid from various biological samples with simple sample collection, room temperature storage and a simple processing technique. To achieve rapid nucleic acid extraction for POCT, we tested the effect of the FTA card drying period (5, 10, 15, 30, 45 and 60 min), *i.e.*, the cell lysis period, on the biosensor. The extraction efficiency was determined by quantitative real-time PCR (qPCR) and LFAs. The data from qPCR showed that a minimum of 15 min was able to achieve a lower cycle threshold (CT) value, which indicates a lower number of cycles required for the fluorescence signal to reach the threshold level, demonstrating a higher number of DNAs being extracted as compared to that of 5 and 10 min (Fig. 2A). To further confirm the result, we performed LFAs. Likewise, we found that 15 min FTA card drying produced a significantly ($p < 0.05$) higher optical density of the test zone as compared to that of 5 and 10 min, indicating that 15 min of cell lysis was able to release a significant amount of DNA for downstream processing (Fig. 2B). Taken together, our results showed that 15 min of FTA card drying allowed sufficient lysis of cells to release a large amount of DNA for amplification and detection. Therefore, 15 min of FTA card drying was selected as the optimum extraction period in the present study.

In fact, following the cell lysis, potential DNA polymerase inhibitors from the biological sample (proteins, cell debris and other components) and chemicals in FTA cards must be removed by the purification reagent as they may potentially denature the polymerase used in the subsequent amplification process. The purification reagent, as a surfactant, could also affect the subsequent amplification process, which therefore must be washed away by the TE buffer.[20] Conventionally, the FTA card is washed with 200 μL of purification reagent and 200 μL of TE buffer for a total of 2–4 washes in tubes prior to the amplification process according to the manufacturer's instruction. To make our integrated biosensor simpler and easier to use, a low volume of buffer with a reduced number of washes is essential. Therefore, we optimized both factors in the extraction process (Fig. 2C). The efficiency of extraction was evaluated by LFA following 1 hour of LAMP. Firstly, following the addition of sample onto the FTA card and 15 min of incubation at room temperature, we optimized the minimal volume of FTA purification reagent and TE buffer required for optimum function of the integrated biosensor. In reagent volume optimization, we used a larger volume of TE buffer to wash away the excess FTA purification reagent that might affect the amplification. The FTA purification reagent and TE buffer volumes tested were 20 μL and 40 μL, 40 μL and 80 μL, and 80 μL and 160 μL,

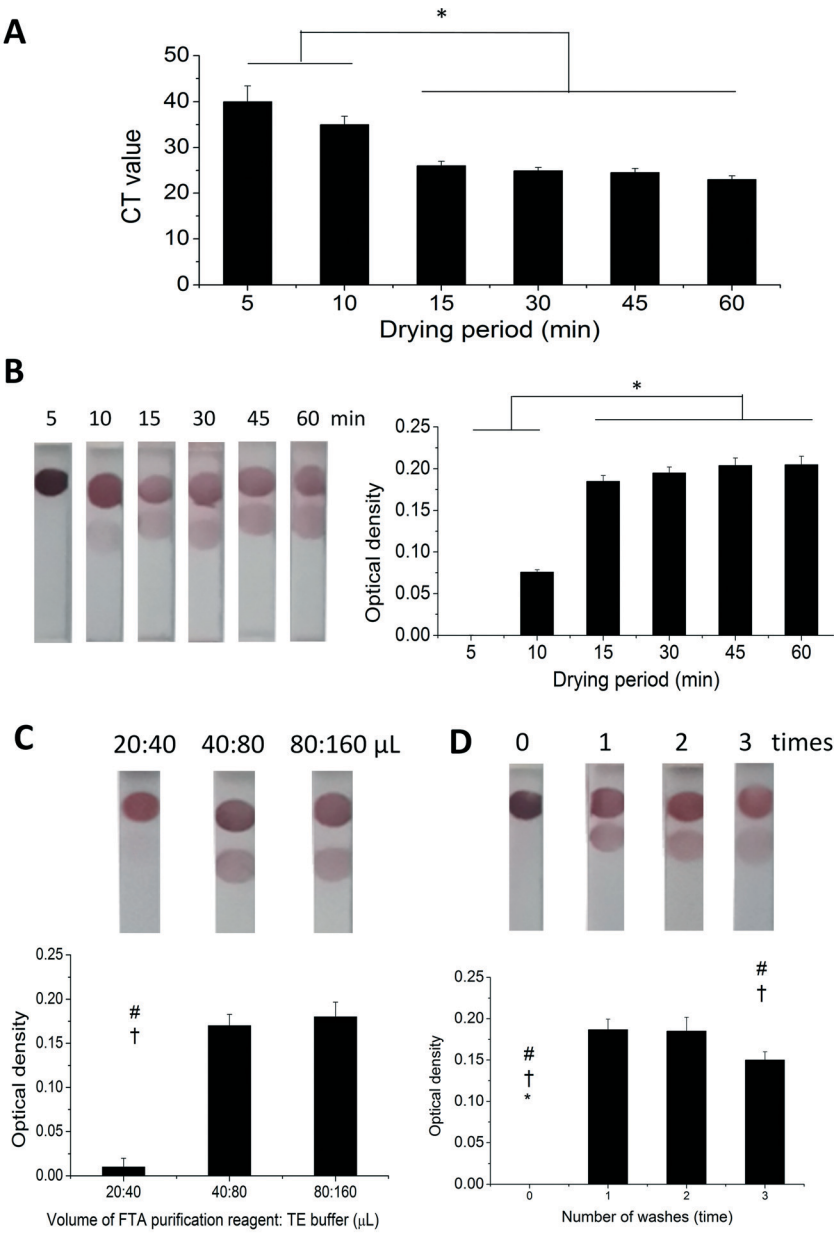

Fig. 2 Optimization of paper-based DNA extraction. A duration of 15 min was selected as the optimum FTA card drying period based on the lower CT value in qPCR (* $p < 0.05$) (A) and the higher optical density in LFA (* $p < 0.05$) (B) as compared to the shorter drying period. (C) A volume of 40 μL FTA purification reagent and 80 μL TE buffer was selected as the optimum wash volume due to the significantly higher optical density of the test zone as compared to that of the lower volume († $p < 0.05$ relative to 40:80, # $p < 0.05$ relative to 80:100). (D) One wash was able to provide a significantly higher optical density than that without washing (* $p < 0.05$ relative to 1 wash, † $p < 0.05$ relative to 2 washes, # $p < 0.05$ relative to 3 washes) (n = 3).

respectively, for a total of two washes, where the volumes were about 10 times lower than that stated in the conventional protocol. We found that 40 μL of FTA purification reagent and 80 μL of TE buffer produced a significantly ($p < 0.05$) higher optical density of the test zone as compared to that of 20 μL purification reagent and 40 μL buffer (Fig. 2C). The volume of 20 μL purification reagent and 40 μL buffer might not be sufficient to fully wash away the inhibitors and chemicals from the FTA card, resulting in a low LFA signal.

With the optimum volume of 40 μL of FTA purification reagent and 80 μL of TE buffer, we investigated the number of washes required to completely wash away the polymerase inhibitors from the FTA cards. We found that there was no significant difference ($p > 0.05$) in optical density of the test zones between one and two washes. However, three washes significantly ($p < 0.05$) reduced the signal of the assay (Fig. 2D), which might be due to the loss of some DNA after being washed with the excess wash volumes.[20] Therefore, we

suggest that one wash is sufficient to completely wash away the inhibitors.

FTA cards are impregnated with a patented chemical formulation for DNA storage, lysis and extraction. As they have been extensively used for DNA collection and storage, we suggest that besides enabling immediate sample processing, our biosensor also allows sample storage in remote settings prior to analysis, which are greatly useful when further laboratory tests are required to confirm the diagnosis, especially for diagnosis of chronic diseases (*e.g.*, cancer). As compared to other available paper-based biosensors,[17,18] which do not allow sample storage, our prototype allows sample storage at ambient temperature by protecting DNA from degradation for downstream analysis,[22] making it a very attractive tool for both onsite sample collection and storage,[23,24] and immediate analysis.

To confirm the paper-based LAMP reaction temperature, LAMP was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Japan) in the integrated biosensor over a range of temperatures (58, 60, 63, 65 and 68 °C) for 60 min in accordance with the manufacturer's instructions. The efficiency of LAMP was determined by electrophoresis and SYBR Green I fluorescence staining. Electrophoresis showed that a temperature of 65 °C produced the most clearly visible bands, indicating the highest efficiency of amplification among all temperatures tested (Fig. S2A†). The result successfully proves that 65 °C is the optimum temperature for the *Bst* DNA polymerase activity in paper-based LAMP, which agrees with the findings in tube-based LAMP.[25,26] In line with the result of electrophoresis, with a LAMP temperature of 65 °C, SYBR Green I staining showed the densest yellowish-green LAMP product under visible light and an increased

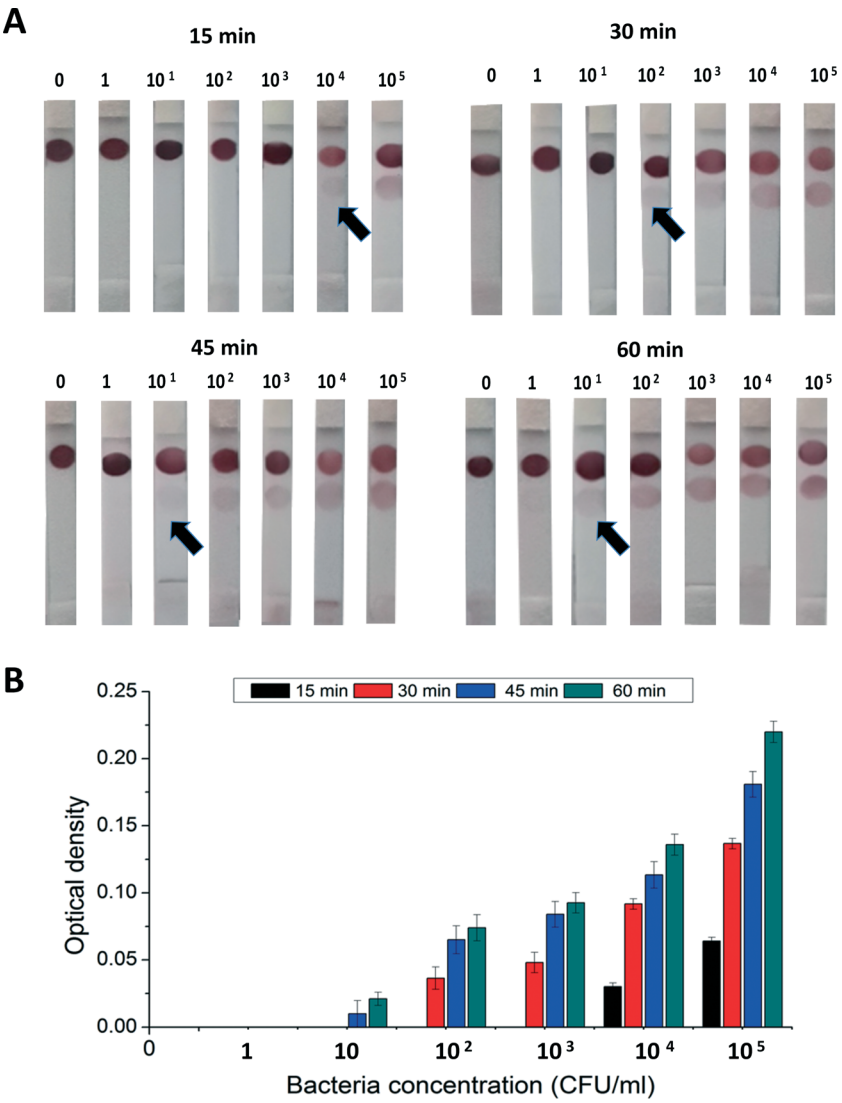

Fig. 3 Optimization of paper-based LAMP. The detection limits of 45 and 60 min amplification periods were lower as compared to 15 and 30 min of amplification. A minimum of 45 min amplification period was able to achieve a detection limit of as low as 10 CFU mL^{-1} bacteria (n = 3) as indicated by the red signal shown at the test zone (3A) and optical density obtained through gray scale analysis of the test zone (3B).

fluorescence signal under UV light (Fig. S2B†) among all temperatures tested, indicating that 65 °C produced more amplicons. Therefore, 65 °C was selected as the optimum LAMP temperature.

To optimize the amplification period in the present study, we performed LAMP in the integrated biosensor at the optimum temperature of 65 °C and a range of incubation times (15, 30, 45 and 60 min) with a serial concentration of *E. coli* (1, 10, 10^2, 10^3, 10^4 and 10^5 CFU mL^{-1}) (Fig. 3). We found that the longer the amplification period, the lower the detection limit of the assay. The detection limits of 45 min and 60 min amplification periods were lower than those with 15 and 30 min of amplification. A minimum of 45 min amplification period was found to be capable of successfully achieving a detection limit of as low as 10 CFU mL^{-1} bacteria, as indicated by the observable red signal in the test zone (Fig. 3A) and the optical density obtained through grey scale analysis of the test zone (Fig. 3B).

To demonstrate the potential use of our biosensor for applications such as medical diagnostics, environmental monitoring and food safety analysis, we tested our biosensor with *E. coli*-spiked whole blood samples, drinking water, milk and spinach over a range of bacteria concentrations (1, 10, 10^2, 10^3, 10^4 and 10^5 CFU mL^{-1}) (Fig. 4).

In the detection of *E. coli* in drinking water, our biosensor achieved a detection limit of as low as 10 CFU mL^{-1} (Fig. 4A), which is similar to that in PBS solution. The data prove that our biosensor can sensitively detect target analytes in contaminated water, which is comparable to existing paper-based assays (10–10^2 CFU mL^{-1}),[27–29] offering great potential in applications of environmental and water safety analyses.

To evaluate the potential use of the biosensor in food safety analysis, we tested it with *E. coli*-spiked milk and spinach samples. Similar to the data of PBS and drinking water, the assay achieved the detection limit of 10 CFU mL^{-1} bacteria in the milk sample (Fig. 4B). Even though milk components (*e.g.*, Ca^{2+}) have been reported to inhibit the amplification process by reducing the exposure of DNA to the polymerase,[30] our results did not show such a negative impact, which further proved the successful removal of the amplification-inhibitory components through the purification and washing process. In the detection of *E. coli*-spiked spinach sample, we found that the detection limit was 10^3 CFU mL^{-1}, which was higher as compared to other samples

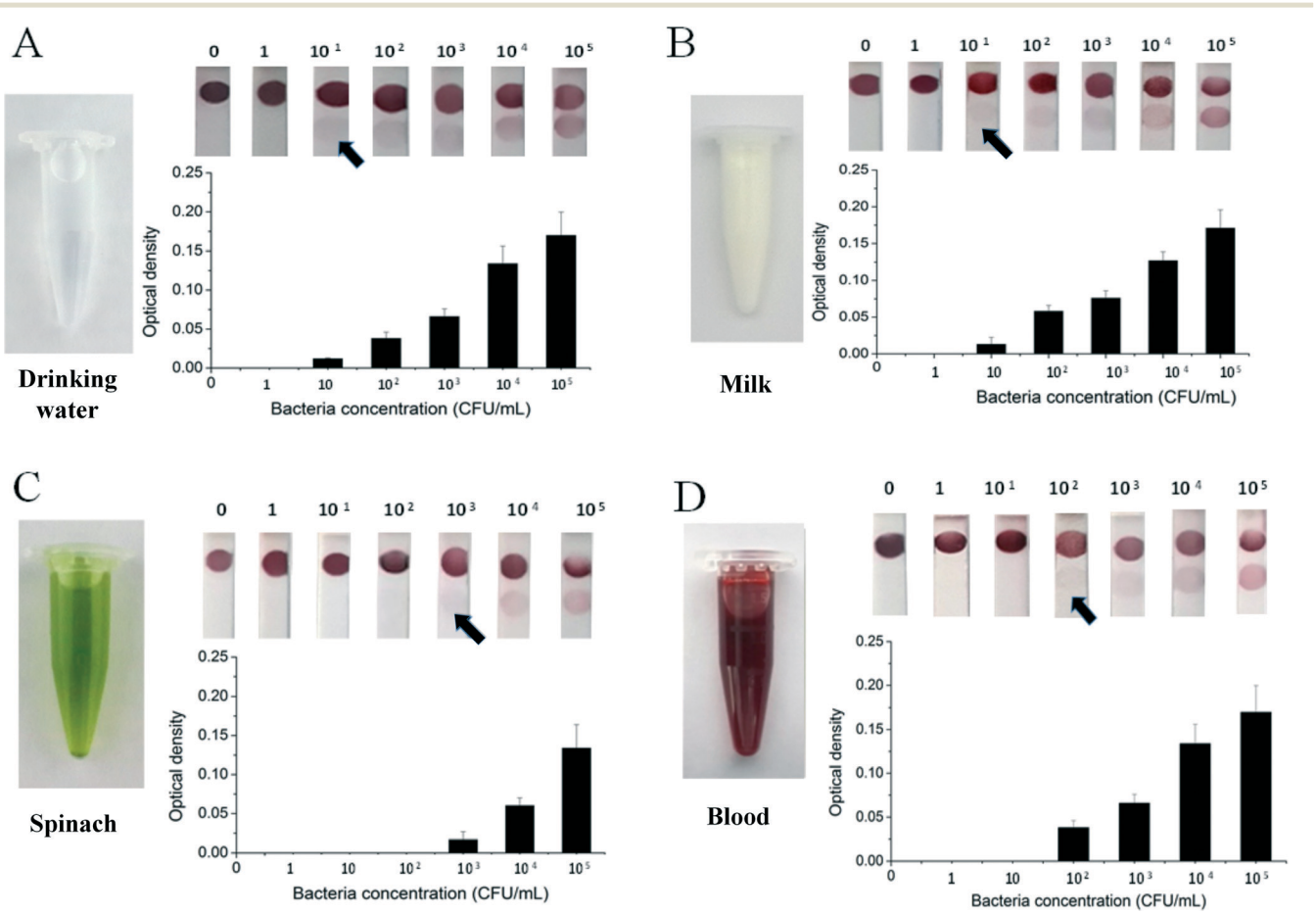

Fig. 4 Integrated paper-based *E. coli* biosensor for various biological sample tests. The integrated *E. coli* biosensor could effectively detect a real sample from drinking water (A), milk (B), spinach (C) and whole blood (D) with a detection limit of 10, 10, 1000, and 100 CFU mL^{-1}, respectively, showing its great potential for future food and water safety analyses and medical diagnostics (n = 3).

(Fig. 4C). This might be due to the requirement for pre-processing steps (*e.g.*, filtration) to remove the residue of spinach leaves prior to the detection, resulting in the loss of bacteria. The ability to detect 10 and 10^3 CFU mL^{-1} *E. coli* in milk and spinach samples, respectively, was comparable or even more sensitive than other existing paper-based assays (~10^3–10^6 CFU mL^{-1});[31,32] considering the simplicity and accuracy of the assay, our all-in-one prototype offers great potential for food and water safety analyses.

On the other hand, sepsis is known as the current leading cause of death.[33] The blood culture method represents the gold standard for determination of sepsis, which is however time-consuming (2–5 days) and highly dependent on skilled operators, thus less suitable for POC applications. Rapid molecular diagnosis is critical for early patient management to reduce the risk of disease transmission. To investigate the potential use of our integrated biosensor in medical diagnosis (especially in sepsis diagnosis), we tested it with an *E. coli*-spiked human whole blood sample (Fig. 4D). We found that the detection limit was 100 CFU mL^{-1}, which was higher as compared to PBS, drinking water and milk sample. This might be due to the presence of a significant amount of white blood cells, which would also be lysed by the FTA card, hence reducing the space available for binding and lysing of bacterial cells. However, with the ability of sensitively detecting *E. coli* in whole blood with comparable or even lower detection limit than conventional assays (10–500 CFU mL^{-1}),[33] our biosensor holds great potential to complement conventional culture-based techniques to achieve rapid, sensitive and specific clinical diagnosis.

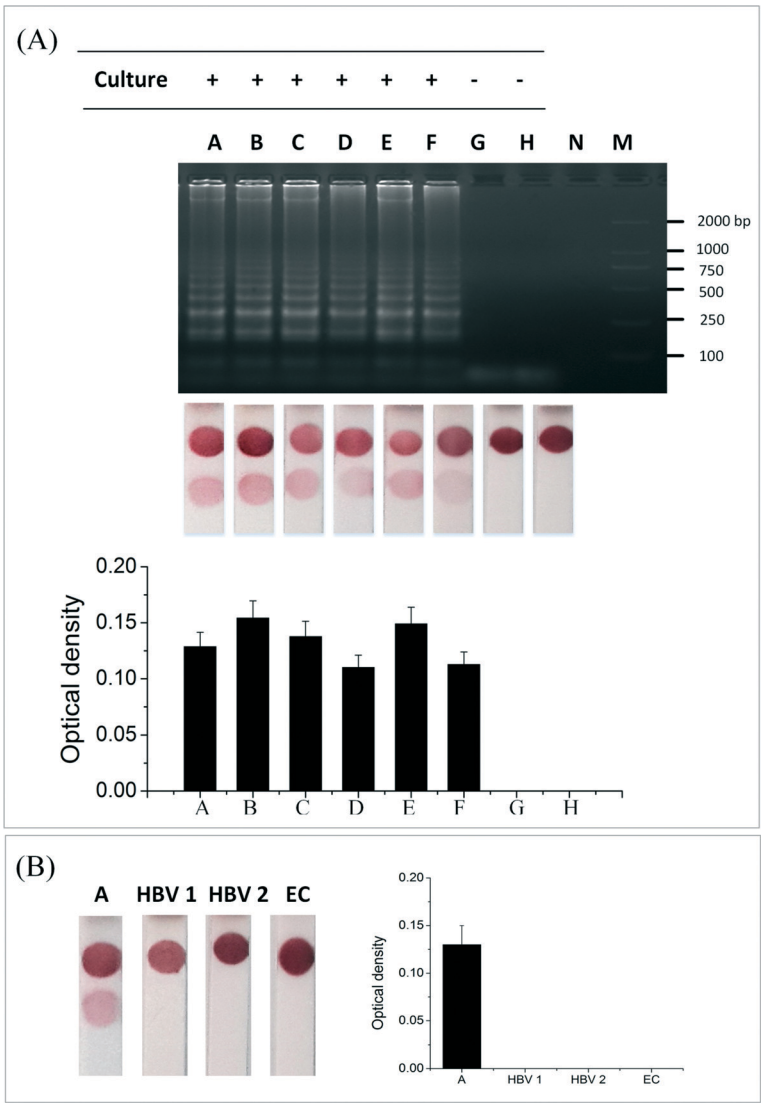

Fig. 5 Clinical sample testing with an integrated paper-based *S. pneumonia* biosensor. In agreement with the result of the gold standard culture method, by using the *S. pneumonia* biosensor, the *S. pneumonia*-positive samples (samples A–F) showed clearly visible bands in electrophoresis, a red signal at the test zone of the strip with a significant optical density as compared to the healthy donor samples (samples G & H) (A) (N = negative control, M = 100–2000 bp marker). Good specificity was demonstrated by the only positive result shown in the *S. pneumonia* sample (sample A) whereas the two HBV samples (HBV 1 and 2) and spiked *E. coli* sample (EC) showed negative results (B).

As there is an urgent need for nucleic acid based-POCT, particularly rapid medical diagnosis in resource-poor settings, we evaluated the ability of the biosensor to perform clinical assessment. To prove the potential use of our prototype for the detection of various targets, another target DNA, *Streptococcus pneumonia* was selected as a model analyte in clinical assessment. Six *S. pneumonia*-positive samples and two *S. pneumonia*-negative samples (confirmed by the gold standard culture method) were further tested with conventional benchtop DNA analysis (tube-based extraction, tube-based LAMP and electrophoresis) prior to the testing using our integrated biosensor. In agreement with the result of the gold standard blood agar culture method, the *S. pneumonia*-positive samples showed clearly visible bands whereas *S. pneumonia*-negative samples showed no bands in electrophoresis (Fig. 5A). Similarly, using the paper-based biosensor, qualitative detection of the positive sample can be done by observing the red signal shown at the test zone with a significantly higher optical density as compared to that of the healthy donor sample (Fig. 5A). Unlike the abovementioned conventional method, our biosensor enables both qualitative and quantitative detection. The biosensor also showed good specificity as indicated by the only positive result shown in the *S. pneumonia* sample, while the other samples (two HBV samples (HBV 1 and 2) and spiked *E. coli*) showed negative results (Fig. 5B). The entire sample-to-answer process requires only 1 h instead of 2–3 days required in conventional bacterial culture.

In the present study, we reported for the first time the integration of nucleic acid extraction, amplification and colorimetric detection into one single paper-based biosensor, which could significantly help in rapid target detection at the POC. The process could be performed with a handheld battery-powered heating device without the need for an external electrical power supply, increasing its portability and use in resource-poor settings. Similar to the "paper-machine" proposed by Whitesides' group, our prototype possesses characteristics such as rapid, low cost and user-friendly. The entire assay can be completed in about 1 h instead of more than 5 h required in conventional assays. Unlike previously reported equipment-dependent paper-based assays,[16–18] our proposed biosensor does not rely on large equipment (*e.g.*, thermal cycler, electric heater, incubator or water bath) for the amplification process, which further demonstrates its potential use in remote settings. Most importantly, by using our prototype, the colorimetric signal can be easily detected by the naked eye without the need for an extra device (*e.g.*, UV lamp), which is deliverable to the end-users, highlighting its advantages over existing fluorescence detection paper-based biosensors.[20,34,35] The entire sample-to-answer process does not require highly-trained and experienced personnel, which makes it highly suitable for home-based or POC testing.

In addition, by performing the LAMP, our prototype permits highly sensitive and specific target detection as compared to other amplification techniques.[36,37] The high sensitivity of the proposed biosensor is demonstrated by its ability to achieve the detection limit of as low as 10 CFU mL^{-1} in *E. coli* detection, which is comparable or even more sensitive than recently reported nucleic acid-based LFAs (~10^2–10^4 CFU mL^{-1}).[38,39] Even though a few studies reported the ability of ultrasensitive detection of *E. coli* in LFAs (~5–10 CFU mL^{-1}), sophisticated off-chip extraction and amplification is required prior to lateral flow detection, hence restricting their use for POC testing.[28] In line with 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),[40] the proposed biosensor holds great potential for detection of a variety of target analytes for application in medical diagnostics, food safety analysis and environmental monitoring.

Conclusion

In short, we propose an integrated paper-based biosensor, which could perform simple nucleic acid extraction, amplification and detection in about 1 h. Our current prototype produces a simple colorimetric signal detectable by the naked eye, eradicating the need for an extra UV source for assay readout. A specially designed handheld heating device is coupled with this biosensor, which eliminates the requirement for large heating systems (*e.g.*, thermal cycler, electric heater, incubator or water bath), making it more suitable for use in remote settings. Our current prototype could be used to detect various target nucleic acids, signifying its great potential for various POC applications in the near future.

To reduce the operation steps, our ongoing work focuses on incorporating fluidic control technologies into the paper-based biosensor with a simple plastic housing, which could program the multistep process by enabling automated sequential delivery of fluid without intermittent technical disruptions. In addition to having simple operation steps, the capability of preserving the biological components in the biosensor at room temperature, particularly by lyophilization, would be another challenge to eliminate the need for laboratory storage units (*e.g.*, refrigerator). Therefore, our current work also focuses on reagent storage on paper and stability maintenance of the biological components during transport and storage. As multiplex detection represents another major demand for integrated paper-based assays, we are also developing an integrated biosensor with the ability to detect multiple targets simultaneously in a single assay which would immensely improve its usability. We envision that our next generation of biosensor will be simple, cost-effective, portable and fully integrated with an automated multiplex sample-in-answer-out capability to facilitate rapid POC diagnostics in resource-poor settings.

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