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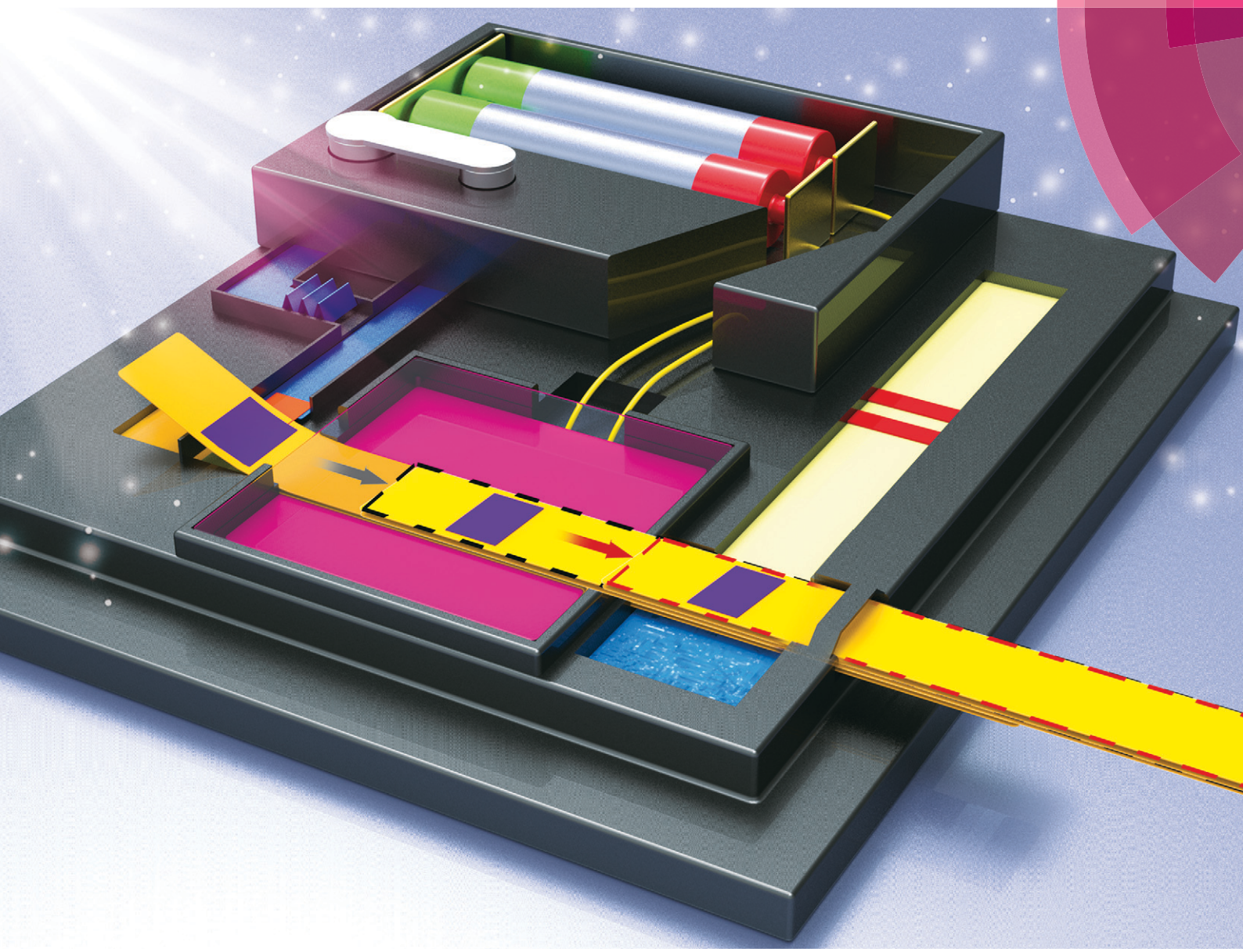

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A fully disposable and integrated paper-based device for nucleic acid extraction, amplification and detection†

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Nucleic acid testing (NAT) has been widely used for disease diagnosis, food safety control and environmental monitoring. At present, NAT mainly involves nucleic acid extraction, amplification and detection steps that heavily rely on large equipment and skilled workers, making the test expensive, time-consuming, and thus less suitable for point-of-care (POC) applications. With advances in paper-based microfluidic technologies, various integrated paper-based devices have recently been developed for NAT, which however require off-chip reagent storage, complex operation steps and equipment-dependent nucleic acid amplification, restricting their use for POC testing. To overcome these challenges, we demonstrate a fully disposable and integrated paper-based sample-in-answer-out device for NAT by integrating nucleic acid extraction, helicase-dependent isothermal amplification and lateral flow assay detection into one paper device. This simple device allows on-chip dried reagent storage and equipment-free nucleic acid amplification with simple operation steps, which could be performed by untrained users in remote settings. The proposed device consists of a sponge-based reservoir and a paper-based valve for nucleic acid extraction, an integrated battery, a PTC ultrathin heater, temperature control switch and on-chip dried enzyme mix storage for isothermal amplification, and a lateral flow test strip for naked-eye detection. It can sensitively detect *Salmonella typhimurium*, as a model target, with a detection limit of as low as 10^2 CFU ml^{-1} in wastewater and egg, and 10^3 CFU ml^{-1} in milk and juice in about an hour. This fully disposable and integrated paper-based device has great potential for future POC applications in resource-limited settings.

1. Introduction

Nucleic acids have been extensively used as molecular biomarkers in various applications such as medical diagnostics,[1–3] food safety control[4,5] and environmental monitoring.[6] Nucleic acid testing (NAT), which generally involves the steps of extraction, amplification and detection, is currently labor-intensive, expensive, time-consuming and equipment-dependent. With increasing incidence of infectious diseases, food and waterborne illnesses, especially in developing areas, point-of-care testing (POCT) has received significantly increasing attention.[3,7–9] Recent advances in paper-based microfluidics make it possible to achieve robust and cost-effective NAT in resource-limited settings. At present, various paper-based devices are being developed to extract nucleic acid from various biological samples,[10] such as commercial filter paper (*e.g.*, FTA card,[11] FTA elute card extraction[12]), the filtration isolation of nucleic acid (FINA) method[13] and paper origami-based extraction.[14] To address the limitations of large equipment-dependent polymerase chain reaction (PCR), several studies have also demonstrated paper-based isothermal amplification by using a water bath or a small external heater, including paper-based loop-mediated isothermal amplification (LAMP),[15] isothermal helicase-dependent amplification (HDA)[16] and recombinase polymerase amplification (RPA).[17] However, off-chip nucleic acid extraction and equipment-based amplification are still required and the amplification and detection have been separately performed, restricting their applications in POC settings.

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To address this, several studies have attempted to integrate nucleic acid extraction, amplification and detection into one single paper-based sample-to-answer device.[18–20] For instance, a "paper machine" has been developed to perform FTA card-based nucleic acid extraction, *in situ* amplification and fluorescent detection by a sliding motion of the device.[21] To achieve simple colorimetric readout, an integrated paper-based sample-to-answer device has been reported combining FTA card-based extraction, glass fiber-based amplification and lateral flow assay (LFA).[20] A fully integrated paper fluidic device has also been developed based on polyethersulfone (PES)-based DNA/RNA extraction, *in situ* amplification and LFA detection.[18,19] These paper-based devices have dramatically reduced the detection time as compared to conventional methods (from ~4 hours to ~1 hour). However, the existing technologies still require off-chip reagent storage (*e.g.*, −20 °C) and complicated operation steps (*e.g.*, adding fluids to multiple parts of the device before extraction and amplification). Besides, the need for external reusable equipment for amplification (*e.g.*, external incubator, water bath or heater) has not only added to the cost and complexity of the assay but also increased the risk of cross-contamination and disease transmission. Hence, there is an urgent need to develop a fully integrated paper-based sample-to-answer device with increased portability and disposability and simple operation steps for nucleic acid testing at the POC.

In this study, we demonstrated a fully disposable and integrated paper-based device with simple user steps for nucleic acid extraction, helicase-dependent isothermal amplification and LFA detection. We utilized a sponge-based reservoir and a paper-based valve to achieve nucleic acid extraction, an integrated battery, a positive temperature coefficient (PTC) ultrathin heater, temperature control switch and on-chip dried enzyme mix storage for isothermal amplification, and a lateral flow test strip for colorimetric signal detection. The device could sensitively detect *Salmonella typhimurium* (as a model analyte) with a detection limit of 10^2, 10^3, 10^3, and 10^2 CFU ml^{-1} in spiked wastewater, milk, juice and egg, respectively. The good specificity of the device was demonstrated by the only positive result shown in the *Salmonella typhimurium* sample whereas other samples (*e.g.*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Escherichia coli*, *Shigella*, *Staphylococcus aureus* and SSC buffer) showed negative results. This fully disposable and integrated device holds great promise for detection of infectious diseases, food contamination and environmental pollution in resource-limited settings.

2. Materials and methods

2.1 Materials

Filter paper and Fusion 5 were purchased from Whatman (Inc., Florham Park, USA). Primers were synthesized from Sangon Biotech (ShangHai) Co., Ltd. IsoAmp® II Universal tHDA kit was purchased from Biohelix (NEB, USA) Co., LTD. XH-RJ202010 ultrathin ceramic PTC heating tablet (20 mm × 20 mm, R = 5 Ω) and SAFTTY BW-BCM 65 °C temperature control switch (15.1 mm × 6.4 mm × 0.3 mm) were purchased from SINHE Electronic Technology (Jiangsu) Co., LTD. Copper sheet (dimension: 90 mm × 8 mm × 0.35 mm) was purchased from a local hardware store. All chemicals used in this study were of analytical reagent grade.

2.2 Bacterial culture

Salmonella typhimurium were cultured on a Luria-Bertani plate and incubated at 37 °C overnight. A single colony was pricked and placed in 10 mL of Luria-Bertani medium and cultured at 37 °C overnight. The concentration of bacteria was measured using a Perkin Elmer Lambda 35 UV/vis spectrophotometer (International Equipment Trading Ltd.). Then, the bacteria were diluted with Luria-Bertani medium to concentrations in the range of 10^0–10^6 CFU ml^{-1}.

2.3 Fabrication of the disposable and integrated device

The fully disposable and integrated device is composed of three functional modules (Fig. 1A), and a housing consisting of one substrate as a supporting base (Fig. 1B) and one top cover (Fig. 1C). The three functional modules, *i.e.*, nucleic acid extraction module, amplification module and LFA detection module, are supported by the substrate (Fig. 1D). The nucleic acid extraction module consists of two sponge-based reservoirs (reservoir 1: washing buffer, reservoir 2: lysis buffer), one paper-based valve, two paper-based channels, one Fusion 5 disk (diameter: 3 mm), and an absorbent pad for waste absorption. The amplification module consists of one PMMA plate as a lid of the amplification module, one PTC ultrathin ceramic heating tablet (20 mm × 20 mm, R = 5 Ω), one 65 °C temperature control switch, two batteries as power supplier (voltage: 3 V), and two layers of copper sheets where the upper layer contains a piece of glass fiber with dried tHDA reagent storage and the bottom layer consists of a Fusion 5 disk connected to the extraction module with a bridge between both layers acting as a separator (Fig. 1B). The LFA detection module contains one test strip and one sponge-based running buffer (reservoir 3). There is a gap that separates the test strip from reservoir 3 prior to nucleic acid detection (Fig. S1A(a)†). The whole device was designed by Solidworks and fabricated using a 3D-printer with photopolymer resin (Formlabs Co., Ltd, USA), Fig. 1E.

2.4 DNA extraction, amplification and detection

DNA was extracted from *Salmonella typhimurium* using the paper-based DNA extraction module of this device. To enhance the efficiency of DNA extraction from the bacteria, the extraction buffer was prepared according to a published protocol[22] with a slight modification. The washing buffer (an initial concentration of 50 mM NaOH) was stored in one sponge with a diameter of 10 mm and a height of 10 mm, which was placed in reservoir 1, while the lysis buffer (an initial concentration of 5% Triton-X 100) was stored in another sponge with a diameter of 8 mm and a height of 10 mm, which was

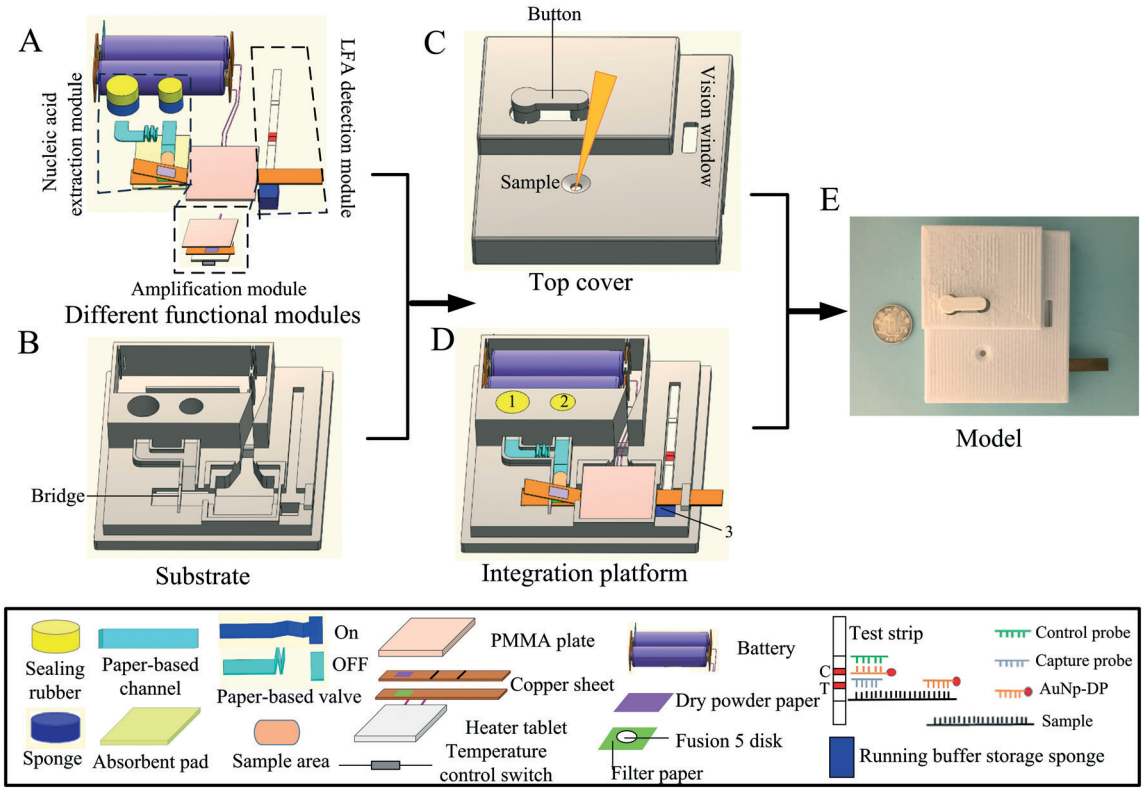

Fig. 1 The schematic of a disposable and integrated paper-based device for nucleic acid extraction, amplification and detection. (A) The different functional modules of the device, including nucleic acid extraction, amplification by the battery heat blocker and LFA detection. (B) The substrate and a bridge that was used to separate the upper layer copper sheet from the bottom layer to prevent the lysis buffer and washing buffer from flowing to the dry powder paper. (C) The top cover of the integrated paper-based device. (D) The integration platform of the different functional modules and the substrate. (E) The model of this device.

placed in reservoir 2. Next, an asymmetric thermophilic helicase-dependent amplification (tHDA) method was utilized for the amplification of the nucleic acid sequence extracted from *Salmonella typhimurium*. The primers were designed from the *fimA* gene sequence of *Salmonella typhimurium* according to the instruction of the tHDA primer design.[23] The details of the primers and probes are stated in ESI† Table S1. The amplification reaction volume was 50 μL, including 5 μL of 10× annealing buffer II, 2 μL of 100 mM $MgSO_4$, 4 μL of 500 mM NaCl, 3.5 μL of IsoAmp dNTP solution, 5 μL of 100 μM forward primer, 1 μL of 5 μM reverse primer, 5 μL of IsoAmp Enzyme Mix and 24.5 μL of ddH_2O. All amplification reagents were freeze-dried and stored in glass fiber (5 mm × 7 mm), which was attached to the upper copper layer (Fig. S1A(d)†). Following the extraction step, the Fusion 5 disk with extracted DNA was moved to the amplification zone that contains a heating tablet. After the amplification, the copper sheet with the amplicons was moved to the detection zone, connecting both the test strip and sponge-based running buffer (reservoir 3) for the detection *via* LFA. After 15 min, the result was observed from the vision window of the detection module by the naked eye. The images of the test line of LFA were captured by an iPhone 6S, and the optical densities of the test strips were measured by the Image-Pro Plus 6.0 software.

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

AuNPs with an average diameter of 13 nm were prepared according to a reported method.[24] AuNPs modified with the *Salmonella typhimurium* detector probe were designed according to the *fimA* gene sequence of *Salmonella typhimurium*.[23] The detailed sequences can be found in ESI† Table S1. The AuNP–detector probe conjugates were prepared according to a reported method with a slight modification.[24]

2.6 Optimization of the disposable and integrated device

To achieve the optimum result, we firstly used an Applied Biosystems Veriti 96 well thermal cycler (USA) to optimize the sample volume, including 10 μL, 30 μL, 50 μL, 70 μL and 90 μL according to our previous studies. Secondly, we optimized the amplification temperature range of tHDA (58, 60, 63, 65, 68 °C) for 90 min. Then, we optimized the reaction time of amplification (30, 45, 60 min). To achieve the cost-effectiveness and integration of this device, we used freeze-dried technology to store the amplification reagents. 1% bovine serum albumin (BSA), 5% Ficoll 400 and 6% Raffinose as protein protectants were respectively used to investigate the effect of the freeze-dried reagents. Meanwhile, 10^4 CFU ml^{-1} of the bacteria was utilized to investigate the storage

effect for one, two and three months. Additionally, we also used an Agilent 34907A data acquisition/Switch Unit (USA) to measure the temperature of the PTC ultrathin ceramic heating tablet at different ranges of voltage (2.8 V, 3.0 V, 3.2 V) for 2 hours. Considering the varied conditions in real applications (different temperatures, different humidities, and different airflows), we tested the temperature of the PTC heating tablet under different conditions with a fixed voltage of 3 V, including different ambient humidities (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% RH) at 20 °C indoor, different ambient temperatures (5, 10, 15, 20, 25, 40 °C) with 40% RH indoor, and different airflows (indoor, outdoor simulated by using a fan) at 20 °C with 40% RH for 2 hours.

2.7 Mathematical simulation

To investigate the heating process using the PTC ultrathin ceramic heating tablet, we performed a mathematical analysis of the natural convective heat transfer in the amplification chamber with two open gates (Fig. 4B(b)). The sizes of the chamber sides (*e.g.*, L_x, L_y and L_z), the heating tablet edges (*e.g.*, a_x and a_y) and the gate width (w) were measured from our device (Fig. S2†). For simplification, the airflow inside the chamber is considered to be incompressible, laminar and Newtonian, and the Boussinesq approximation is imposed on the density of the air.[25] The air density is given as follows,

$$\rho = \rho_0(1 - \beta\cdot(T - T_0)) \quad (1)$$

where T_0, β, and ρ_0 are the room temperature, the volume expansion coefficient and the air density corresponding to T_0, respectively. The governing equations coupled with conservation of mass, momentum and energy equations are used to deal with this heat transfer problem and can be expressed as,

$$\mathrm{div}(\rho\vec{u}) = 0 \quad (2)$$

$$\nabla\cdot(\rho\vec{u}u) = \nabla\cdot(\mu\mathrm{grad}u) - \frac{\partial p}{\partial x} \quad (3)$$

$$\nabla\cdot(\rho\vec{u}v) = \nabla\cdot(\mu\mathrm{grad}v) - \frac{\partial p}{\partial y} \quad (4)$$

$$\nabla\cdot(\rho\vec{u}w) = \nabla\cdot(\mu\mathrm{grad}w) - \frac{\partial p}{\partial z} - \rho g \quad (5)$$

$$\nabla\cdot(\rho\vec{u}T) = \mathrm{div}\left(\frac{\lambda}{c_\mathrm{p}}\mathrm{grad}T\right) \quad (6)$$

where u, v and w are the velocity in the direction of x, y, and z, respectively; μ, p, λ, and c_p are the viscosity, pressure, thermal conductivity and specific heat of air, respectively. The heating tablet provides a constant surface temperature (T_h). The pressures at the two open gates are equal to the atmospheric pressure. Adiabatic and non-slip boundary conditions are applied to the remaining bounding walls. All the parameters used in the modeling are listed in Table S2.† The geometrical model of the square chamber was built with a non-uniform grid by the GAMBIT software, while the mathematical model was solved by using the FLUENT software.

2.8 Testing of various biological samples and the specificity of *Salmonella typhimurium* LFA

We spiked *Salmonella typhimurium* into wastewater, milk, juice and egg samples with a concentration range from 10^0 to 10^6 CFU ml^{-1}. Wastewater was obtained from sewage, and milk, juice and egg were purchased from a grocery store. Additionally, we used 100 nM of the sequences of *Salmonella typhimurium*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Escherichia coli*, *Shigella*, *Staphylococcus aureus* and SSC buffer to detect the specificity of the *Salmonella typhimurium* LFA method.

2.9 Statistical analysis

One-way ANOVA was used to compare the data among different groups. $P < 0.05$ was reported as statistically significant.

3. Results and discussion

3.1 Design of the fully disposable and integrated paper-based sample-to-answer device

To overcome the drawbacks of the existing nucleic acid testing technologies (*e.g.*, need external reusable amplification equipment, complex operations, off-chip reagent storage), we developed a fully disposable and integrated paper-based sample-to- answer device for nucleic acid testing (Fig. 1). The device is composed of three functional modules (Fig. 1A) and a housing containing one substrate (Fig. 1B) and one top cover (Fig. 1C). The three functional modules were developed for nucleic acid extraction, amplification and LFA detection, respectively. The integration platform as shown in Fig. 1D contains the three functional modules supported by the substrate, and the top cover was then mounted to the integration platform to create a fully integrated device (Fig. 1E).

The nucleic acid extraction module is composed of two sponge-based reservoirs (one lysis solution reservoir and one washing solution reservoir), two paper-based channels (one lysis channel and one washing channel), one paper-based valve and one Fusion 5 disk (Fig. 1A). Two sponges were separately placed in the two reservoirs to store the lysis buffer and washing buffer, respectively. Meanwhile, two sealing rubbers connected to the button were placed on top of the sponge to prevent reagent evaporation (Fig. 1A). The button was used to turn on the nucleic acid extraction process by allowing the buffer to flow from the reservoirs to the two

paper-based channels (*i.e.*, sample lysis channel and washing channel) through their respective small holes (Fig. S1A(a)†). Briefly, one end of the lysis channel is connected with the small hole of the sponge-based lysis buffer whereas the other end is connected to the Fusion 5 disk. The Fusion 5 disk is composed of silica-based glass fiber, and it absorbs DNA based on the combination of high affinity between the negatively charged DNA and the positively charged glass fiber.[26] The absorbent pad is placed under the Fusion 5 disk for waste absorption (Fig. S1A(b)†). One end of the washing channel (with "∟" shape) is connected to the small hole of the sponge-based washing buffer, while the other end is connected to the paper-based valve to control fluid flow through the paper-based channel. The integrated copper sheets consist of two layers (Fig. 1A), the upper layer (amplification layer) consists of a piece of glass fiber that stores the dried amplification reagent which will be further described in the next paragraph, whereas the bottom layer consists of a Fusion 5 disk for nucleic acid extraction. During the nucleic acid extraction process, both layers are separated by a bridge (Fig. S1A(c)†) to prevent the extraction reagent from flowing into the amplification layer, which may affect the entire sample-to-answer process.

The nucleic acid amplification module is composed of two batteries, one ultrathin ceramic heating tablet, one 65 °C temperature control switch, two layers of copper sheets and one PMMA plate. The batteries were mounted on the base substrate to power the heating tablet. The 65 °C temperature control switch was mounted on the back of the PTC heating tablet to control the temperature of the PTC heating tablet. During the heating process, the temperature switch will automatically disconnect when the temperature of the PTC heating tablet is higher than 65 °C. The aforementioned copper sheets were placed between the heating tablet and the PMMA plate (Fig. S1A(d)†), where the PMMA plate was used to prevent sample evaporation and cross-contamination. A small piece of insulation pad was integrated into the device, which acts as a "switch" that isolates the battery power source from the electrical circuit prior to nucleic acid amplification (Fig. S1A(e)†). Briefly, following the nucleic acid extraction, the extracted nucleic acid was attached to the Fusion 5 disk, which was used for the subsequent amplification process. The insulation pad was then removed to switch on the power supply for the heating tablet. During the amplification process, the two copper sheets were moved to the amplification zone between the PTC ultrathin ceramic heating tablet and the PMMA plate (Fig. 2B).

The nucleic acid detection module contains one lateral flow test strip and one sponge-based running buffer. Prior to nucleic acid detection, the test strip and running buffer were separated by a gap (Fig. S1A(a)†). When the two copper sheets were moved to the detection zone, it occupied the gap and connected both the test strip and sponge-based running buffer. The buffer and sample automatically flowed into the test strip as driven by the capillary force. In the whole device, the two copper sheets were used to connect the extraction module, amplification module and LFA detection module. The upper layer was labeled with two lines, amplification line and detection line, to indicate the position of both amplification and detection zones for user convenience (Fig. 2B).

Considering that the capacity and life of the battery could affect the final temperature, in this device, we used new batteries to provide the power for the PTC heating tablet during the amplification process. After the amplification, the battery voltage was stable 3 V as measured by a multimeter, indicating that the battery can be also reused for other household appliances to avoid the waste and high cost. Additionally, the PTC heating tablet can also be recycled and used in the device again. But other components (*e.g.*, paper-based channel, reagents and LFA test strip) cannot be used again.

3.2 The whole operation process

In this study, we used detergent-based and alkaline extraction methods to extract nucleic acid, asymmetric tHDA to amplify the nucleic acid and LFA to detect the target. Although Triton X-100 is not necessarily lytic to bacteria, TritonX-100 and NaOH can be used to isolate DNA from blood according to a reported study.[22] Hence, considering the universality of this device in the future, in this study, we also used TritonX-100 and NaOH for DNA extraction from bacteria. As compared to the reported study,[22] in our device, 5% of TritonX-100 and 50 mM NaOH as the initial concentrations of the extraction buffer were respectively utilized to lyse the sample and wash away the debris and PCR inhibitors, because the efficiency of DNA extraction has been shown to increase with increasing concentration of the extraction reagent.[27] The Fusion 5 disk and dry powder paper were separately used to capture the DNA and store the dried tHDA reagents, whereas the lateral flow test strip was utilized to detect the amplicons. Compared to the conventional process (Fig. 2A), the whole operation process of our device is much simpler and only requires a few steps, including sample addition, nucleic acid extraction, removal of the insulation pad, amplification and LFA detection (Fig. 2B, ESI† Movie S1). The detailed process is as follows (Fig. 2C): firstly, the sample was added into the sample area by manual pipetting or dropping. Secondly, the start button was manually pressed to start the extraction process, where the lysis buffer firstly flowed into the paper-based channel *via* small holes (Fig. S1A(a)†) and reached the sample area to lyse the sample. Then, the washing buffer activated the paper-based valve on the washing channel to connect to the lysed channel and flowed through both the sample area and Fusion 5 disk. The Fusion 5 disk was used to capture the DNA, which was connected to an absorption pad for waste removal (the whole extraction is shown in Fig. S3†). Following the DNA extraction, the two copper sheets that consist of the Fusion 5 disk with the DNA template and glass fiber with tHDA reagents were simultaneously moved to the amplification zone (Fig. 2B), and the insulation pad was removed to start the amplification process. Finally, after the

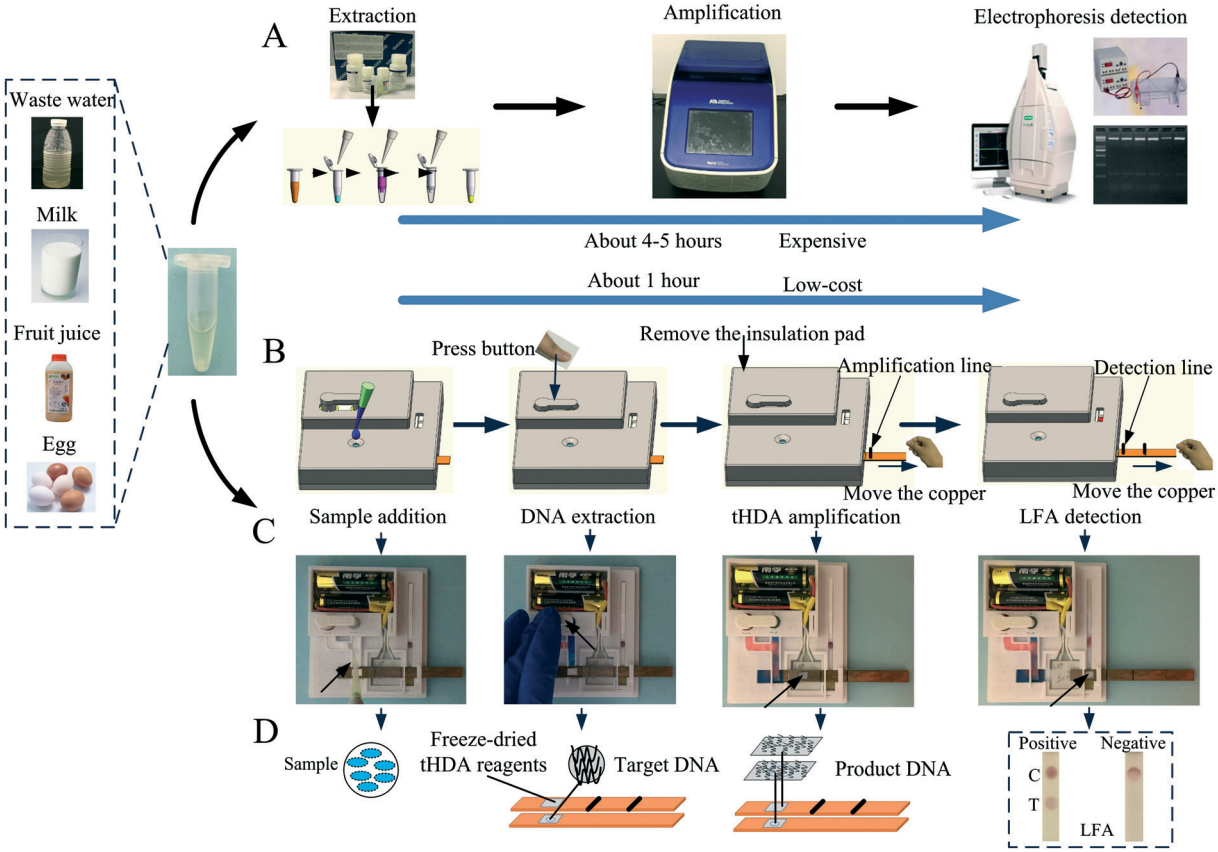

Fig. 2 The whole operation process of the fully disposable and integrated paper-based sample-to-answer device. Samples from wastewater, milk, fruit juice and egg. (A) The conventional operation process of nucleic acid extraction, amplification and detection. (B) Schematic diagram of the operation of the disposable and integrated paper-based sample-to-answer device, including sample addition, DNA extraction, tHDA amplification and LFA detection. (C) The internal diagram of the device with different steps. (D) The schematic diagram of the main parts of each step.

amplification, the copper sheets were moved to the LFA detection module (Fig. 2B), and the target and running buffer flowed into the test strip as driven by the capillary force. The colorimetric signals produced by LFA were then observed by the naked eye.

3.3 Optimization parameters of the disposable and integrated device

To obtain the optimum detection result, we optimized the sample volume, amplification temperature and amplification period used for the device using a fixed diameter of Fusion 5 disk (diameter of 3 mm) (Fig. 3). We observed that with 10 μL of sample, the optical density of the test zone was significantly lower than other sample volumes (30 μL, 50 μL, 70 μL, 90 μL), Fig. 3A. There was no significant difference in optical densities of test zones using 30 μL, 50 μL, 70 μL and 90 μL of samples, suggesting that the Fusion 5 disk with a diameter of 3 mm had the maximum DNA capturing capability when the sample volume was 30 μL. This result was further confirmed by the electrophoresis test, where we observed the higher optical density of the band for the case of 30 μL, 50 μL, 70 μL and 90 μL (Fig. S1B†). Since the temperature may affect the amplification efficiency, we also investigated the effect of amplification temperature (58 °C, 60 °C, 63 °C, 65 °C, 68 °C) on LFA with a 60 min amplification period, Fig. 3B. We found positive results for 63 °C, 65 °C and 68 °C as reflected by the red color shown in the test zone, but not for 58 °C and 60 °C. By comparing the optical density of the test zone at different temperatures, the color and optical density of the test zone of 65 °C was significantly higher than those of 63 and 68 °C (Fig. 3B), indicating that the optimum amplification temperature was 65 °C, which was further confirmed by the electrophoresis result (Fig. S1C†). Furthermore, we investigated the effect of the amplification period (30 min, 45 min and 60 min) on the detection limit of bacteria in PBS (Fig. 3C). We found that the result was negative for the case of 30 min, the detection limit for 45 min was 10^6 CFU ml^{-1}, while the detection limit for 60 min was significantly improved to 10^2 CFU ml^{-1}. This result indicated that the optimum amplification period was 60 min.

To increase the portability of the device and eliminate the cold-chain requirement in resource-limited settings, we stored the tHDA amplification reagents on the device by using the freeze-drying method. To evaluate the reagent preservation effect of different protein protectants for POC use, we added different protein protectants (1% bovine serum albumin (BSA), 5% Ficoll 400 and 6% Raffinose) to the tHDA

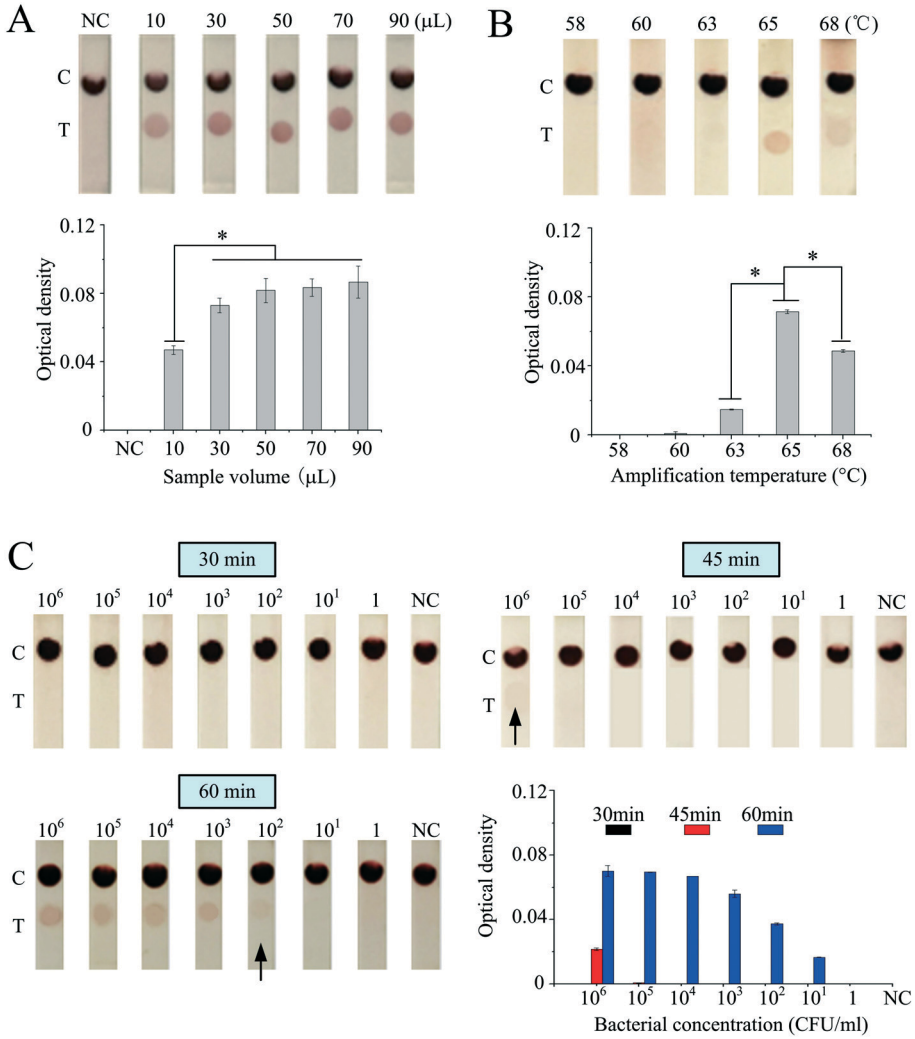

Fig. 3 Optimization of extraction and amplification parameters. (A) Different sample volumes, 10 μL, 30 μL, 50 μL, 70 μL, 90 μL, were used for optimizing the extraction parameter, (B) different temperatures, 58 °C, 60 °C, 63 °C, 65 °C and 68 °C, were utilized for optimizing the tHDA amplification parameter, (C) different times, 30 min, 45 min and 60 min, were utilized for optimizing the amplification period of our device (NC – negative control).

reaction reagent, which was then stored by the freeze-drying method. We found that the result of 1% BSA was significant better than those of 5% Ficoll 400 and 6% Raffinose based on the color and optical density of the test zone (Fig. S1D†). The stabilization was probably due to the hydrogen bonding between BSA and the dried proteins.[28] To evaluate the stability of on-chip reagent storage at different storing periods and temperatures for future POC applications, we evaluated their performance after one-, two- and three-month storage at different temperatures (−20 °C, 4 °C, 25 °C, 37 °C, 45 °C, 56 °C). We vacuum-packed the disposable and integrated device after placing the lysis and washing buffers in the sponges and drying tHDA reagent on paper. We observed that after one month of storage, the positive signal shown by the samples from −20 °C was significantly higher than those from 4 °C and 25 °C, whereas after two months of storage only the samples from −20 °C and 4 °C showed a positive result, which showed a negative result for three months of storage. Other temperatures (37, 45, 56 °C) showed negative results after one to three months of storage, which may be due to the reduced protein activity with increasing temperature[29] (Fig. 4). To achieve room temperature storage and avoid the need for external storage unit (*e.g.*, fridge) during shipping or in POC settings, further optimization would be performed in the future in collaboration with a commercial partner.

To avoid the use of external reusable amplification equipment and improve the portability of the device, we utilized the PTC heating tablet and temperature control switch combined with the battery to provide the optimum amplification temperature. During the heating process, the PTC heating tablet, as a kind of self-regulating heating element, was utilized for performing tHDA amplification because the fixed resistance PTC heating tablet is an automatic constant temperature device.[30] The temperature switch was used to control the PTC heating tablet when the temperature of the PTC heating tablet was higher than 65 °C. The PTC heating tablet could

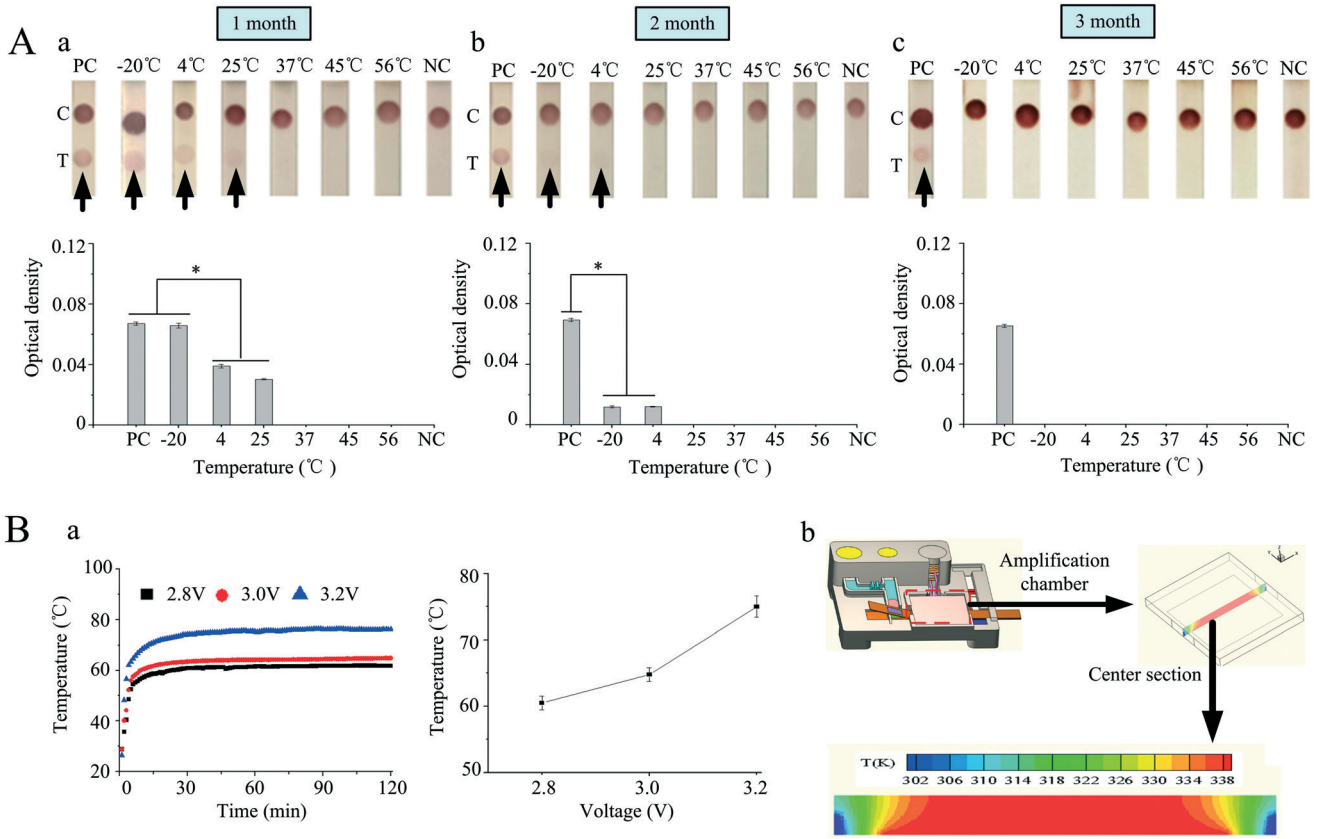

Fig. 4 Optimization of on-chip reagents' storage period and ultrathin heater tablet temperature parameter. (A) The stability test result of tHDA mixes at different storage temperatures and times: (a) one month, (b) two months, (c) three months. (B) (a) Temperature stability of the ultrathin heater tablet inside the device with different voltages, (b) the simulation temperature field of the amplification chamber (PC – positive control, NC – negative control).

provide different temperatures through adjusting the voltages and we achieved a stable condition at 65 °C up to 2 hours when the voltage was 3 V (Fig. 4B(a)). Considering the varied conditions in real applications, we also checked the temperature of the PTC heating tablet with a fixed voltage of 3 V across different ambient conditions of humidity, temperature and airflow (Fig. S4†). We found that the temperature of the PTC heating tablet was stable at 65 °C for all conditions of humidity except 20% RH (Fig. S4A†). Similarly, the PTC temperature was stable at 65 °C when the ambient temperature was in the range of 10–40 °C (Fig. S4B†). However, the PTC temperature was unstable under simulated outdoor airflow conditions (Fig. S4C†). At present, this device prototype is not yet ready for use anywhere except in the range of humidities (30–90% RH), temperatures (10–40 °C) and indoor condition. To further expand the application range of this device, our future work would focus on development of a disposable and portable device with stable environmental conditions to provide an optimum condition for nucleic acid-based testing.

To investigate the temperature of the chamber during the heating process using the PTC ultrathin ceramic heating tablet, we also performed a numerical analysis of the temperature field in the amplification chamber by using the FLUENT software. From the result, we found that the temperature of the main area of the amplification chamber remains stable at 65 °C though the temperature of the two open gates of the amplification chamber is less than 65 °C (Fig. 4B(b)). The simulation results indicate that the isothermal amplification could be well performed without considering the position error of the copper sheet.

3.4 Testing of various biological samples

To verify the potential application of our fully disposable and integrated paper-based sample-to-answer device for POC testing, different concentrations of *Salmonella typhimurium* ranging from 10^0 to 10^6 CFU ml^{-1} were spiked into different samples, including wastewater, milk, juice and egg. After testing with the paper-based device, we found that the detection limit of bacteria was 10^2, 10^3, 10^3, 10^2 CFU ml^{-1} in wastewater, milk, juice and egg, respectively (Fig. 5), which is similar to a reported paper-based device with a detection limit of 10^3 CFU ml^{-1}.[31] As for wastewater and egg, the detection limit was similar with that of PBS, which was lower than those of bacteria in milk and juice. This is because milk and juice contain some inhibitors (*e.g.*, protease, calcium ions and additives) which can reduce the amplification efficiency. Additionally, we also used 100 nM of the sequence of *Salmonella*

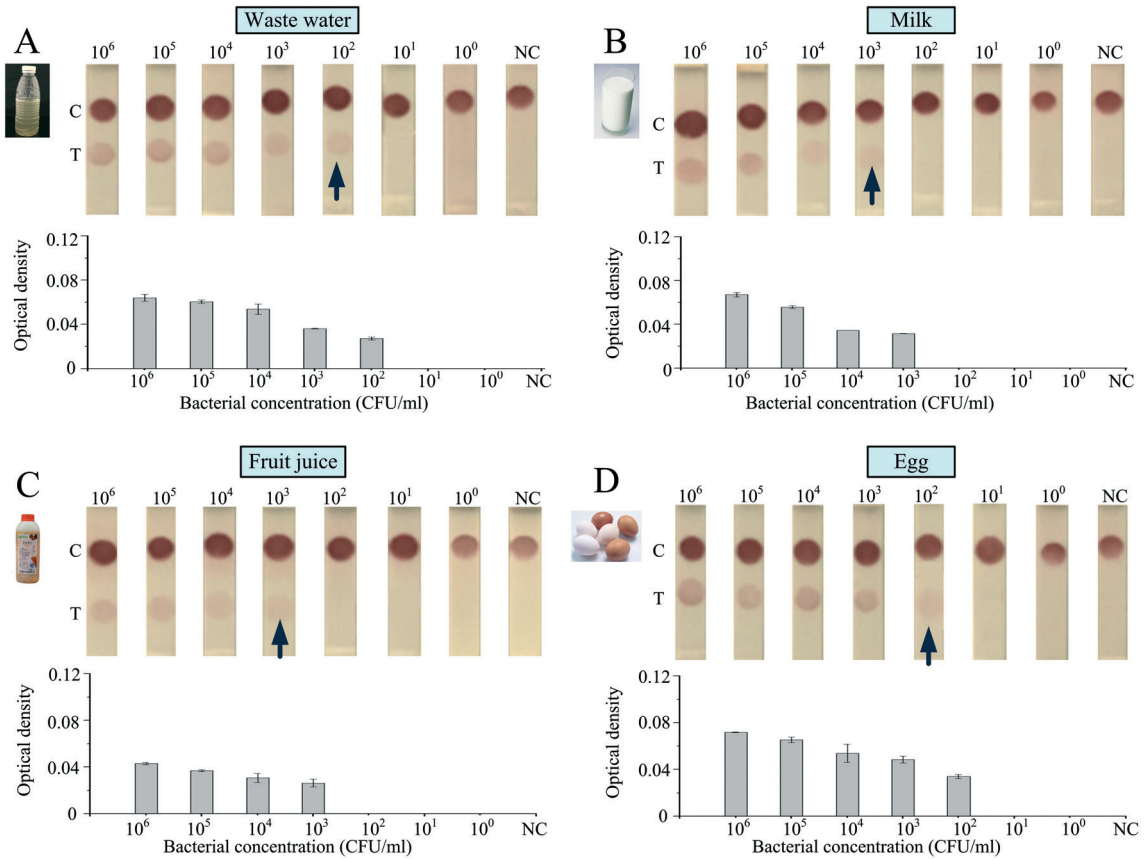

Fig. 5 Fully disposable and integrated paper-based device for various biological sample tests. *Salmonella typhimurium* from 10^0 to 10^6 CFU ml^{-1} were spiked into (A) waste water, (B) milk, (C) juice and (D) egg and utilized for the validation of this assay with a detection limit of 10^2, 10^3, 10^3, 10^2 CFU ml^{-1}, respectively (NC – negative control).

typhimurium, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Escherichia coli*, *Shigella*, *Staphylococcus aureus* and SSC buffer to verify the specification of the *Salmonella typhimurium* LFA method, which showed a positive result. Other investigations showed negative results (Fig. S1E†), indicating the good specificity of our device.

We successfully demonstrated that our prototype could achieve sample-in-answer-out testing using simple operations, which could be performed by untrained users. To achieve the simple and integrated nucleic acid testing, in this study, we developed a paper-based DNA extraction module in the integrated device. As compared to the existing integrated paper-based device that combines FTA card-based extraction, glass fiber-based amplification and LFA,[20] our device solved the extraction and amplification reagents' storage problem. As compared to the fully integrated paper fluidic device based on polyethersulfone (PES)-based DNA/RNA extraction, *in situ* amplification and LFA detection,[18,19] our device achieved equipment-free amplification using an on-chip heating tablet. Additionally, when the bacteria target was directly used for tHDA amplification, we found that the results were negative because the inhibitor of the bacteria sample could affect the tHDA amplification. On the other hand, we also used a simple filtration method to collect the bacteria and then removed inhibitors by washing, which gave positive results. However, the simple filtration method and washing steps still need external multiplex operations, increasing the complexity of nucleic acid testing. Furthermore, our device decreases the cost, reduces the operation steps and realizes the fully portable and disposable feature. Thus, our device offers great potential to meet the ASSURED criteria suggested by WHO for nucleic acid testing in POC settings.

4. Conclusion

In summary, we developed a fully disposable and integrated paper-based device by integrating paper-based nucleic acid extraction, paper-based isothermal amplification and LFA into one paper device, and achieved nucleic acid testing in about an hour. This device only needs simple operations for the entire sample-to-answer nucleic acid testing, including adding the sample, pressing the button to begin the nucleic acid extraction, moving the copper sheet to the amplification zone for tHDA amplification and moving the copper sheet to the detection zone for LFA detection. This prototype could be directly utilized in resource-limited settings without special training, external equipment (*e.g.*, thermal cycler, refrigerator) and complex operation. In comparison with existing integrated paper-based sample-to-answer devices, our device

further decreases the cost, reduces the operation step and realizes the fully portable and disposable feature.

According to reported studies and our study, TritonX-100 and NaOH can be used for blood sample and bacteria sample testing. To further enhance the universality of this device, our ongoing work would focus on extracting nucleic acid (*e.g.*, DNA or RNA) from various biological samples such as Gram positive bacteria, cells, viruses and so on, which can further expand the application of this device at the point-of-care. We envision that this disposable and integrated paper-based device would be a powerful tool for nucleic acid testing in resource-limited settings.

Declaration of interest

The authors declare that they have no conflict of interest.

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