Multiplexed colorimetric detection of SARS-CoV-2 and other pathogens in wastewater on a 3D printed integrated microfluidic chip

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ABSTRACT

Severe acute respiratory coronavirus 2 (SARS-CoV-2) pandemic has become a global public health emergency. The detection of SARS-CoV-2 and human enteric pathogens in wastewater can provide an early warning of disease outbreak. Herein, a sensitive, multiplexed, colorimetric detection (termed "SMCD") method was established for pathogen detection in wastewater samples. The SMCD method integrated on-chip nucleic acid extraction, two-stage isothermal amplification, and colorimetric detection on a 3D printed microfluidic chip. The colorimetric signal during nucleic acid amplification was recorded in real-time and analyzed by a programmed smartphone without the need for complicated equipment. By combining two-stage isothermal amplification assay into the integrated microfluidic platform, we detected SARS-CoV-2 and human enteric pathogens with sensitivities of 100 genome equivalent (GE)/mL and 500 colony-forming units (CFU)/mL, respectively, in wastewater within one hour. Additionally, we realized smart, connected, on-site detection with a reporting framework embedded in a portable detection platform, which exhibited potential for rapid spatiotemporal epidemiologic data collection regarding the environmental dynamics, transmission, and persistence of infectious diseases.

1. Introduction

According to a World Health Organization report, the novel coronavirus (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), resulted in over 70 million confirmed cases and more than 1.6 million deaths worldwide by December 15, 2020 [1]. Recent reports have shown that SARS-CoV-2 is present in wastewater environments (e.g., wastewater, sludge), which offers an opportunity to trace COVID-19 sources with wastewater-based epidemiology [2–6]. Combined with clinical data, wastewater-based epidemiological information may enable critical monitoring of SARS-CoV-2 transmission and early indications of COVID-19 outbreaks regardless of symptoms [7–9]. In addition, the infectious disease caused by human enteric pathogens (e.g., *Salmonella typhimurium*) in fecally contaminated water has emerged as a global health concern, influencing 9% of the world's population. [10–13] Early monitoring of SARS-CoV-2 and human enteric pathogens in wastewater plays a crucial role in disease transmission tracking, epidemiological studies, and environmental surveillance. Thus, there is an unmet need for a simple, rapid, sensitive, and field-portable method for detecting SARS-CoV-2 and human enteric pathogens in wastewater environments.

Nucleic acid amplification testing (NAAT) provides rapid, specific, and sensitive pathogen detection. The polymerase chain reaction (PCR) is the most widely used NAAT method for detecting and identifying pathogens. However, PCR typically requires an expensive thermal cycler and highly trained personnel; thus, PCR is restricted to centralized laboratories and is not suitable for on-site detection [14,15]. The development of several isothermal amplification methods, such as recombinase polymerase amplification (RPA), and loop-mediated isothermal amplification (LAMP), has led to attractive alternatives for rapidly amplifying target nucleic acids with the advantages of simplicity, speed and cost-effectiveness [13,16,17]. However, to meet the stringent limit-of-detection specifications required for pathogen detection, it is necessary to enrich pathogens and extract their nucleic acids from large-volume samples before nucleic acid amplification detection can be performed [18]. Nucleic acid sample preparation

remains a big challenge in the development of integrated molecular detection systems for on-site detection. To simplify nucleic acid sample preparation, researchers have developed a syringe filter-based method to extract pathogen nucleic acid from wastewater by employing magnetic bead separation technology [19,20]. However, this process requires a relatively long time and involves multiple manual operations, which is not ideal for rapid on-site detection.

Integrated microfluidic technology offers outstanding advantages including low cost, high speed, low reagent consumption, and, most importantly, integrated automation of several processes ranging from nucleic acid sample preparation to detection in a single microfluidic chip [21]. However, most current fabrication methods (e.g., soft lithography, hot embossing) are not suitable for fabricating integrated microfluidic chips with complex 3D microstructures due to challenges in accurate fabrication and reliable bonding [22,23], which severely restrict the development of complex integrated microfluidic chips [24]. Fortunately, 3D printing technology has offered rapid, one-step fabrication of integrated microfluidic chips with complex microstructures [25].

In this study, a sensitive, multiplexed, colorimetric detection (termed "SMCD") method was established for rapid detection of SARS-CoV-2 and human enteric pathogens (e.g., *Salmonella typhimurium*) in wastewater using an integrated 3D printed microfluidic chip. We integrated a Flinders Technology Associates (FTA) membrane in the microfluidic chip for nucleic acid sample preparation. We applied the nucleic acid extracted by the FTA membrane directly as a template for on-chip nucleic acid amplification. To enable sensitive and multiplexed detection of pathogens, we combined recombinase polymerase amplification (RPA) and synergetic enhanced colorimetric, loop-mediated isothermal amplification (SEC-LAMP) into the integrated microfluidic chip. The colorimetric signal produced during the SEC-LAMP can be directly read by the naked eye or monitored in real-time by our smartphone-based detection platform without the need for complicated equipment. By taking advantage of smartphone connectivity, test results and locations could be reported on a custom website, enabling smart and connected contamination source tracking. Using our portable detection platform, we have demonstrated multiplexed detection of SARS-CoV-2 and human enteric pathogens in river water samples and municipal wastewater samples.

2. Materials and methods

2.1. Materials and reagents

KCl, KOH, $(NH_4)_2SO_4$, Tween 20, Eriochrome black T (EBT), poly (vinyl alcohol) (PVA), Luria Bertani (LB) broth medium, LB agar medium, brain heart infusion (BHI) broth medium, Whatman FTA membrane, FTA purification reagent and Whatman filter paper were purchased from Sigma-Aldrich. *Bst* 2.0 DNA polymerase (8000 U/mL), $MgSO_4$ and deoxynucleotides (dNTPs) were purchased from New England BioLabs. Hydrophobic polytetrafluoroethylene (PTFE) membrane filter was purchased from Sterlitech Inc. EvaGreen dye was purchased from Biotium. TwistAmp Basic kit for RPA amplification was obtained from TwistDx. Primers were purchased from Integrated DNA Technologies, Inc. DNeasy Blood and Tissue kit for bacterial DNA extraction was purchased from QIAGEN. Acrylics was purchased from IPS Corporation. Clear resin (GPCL02) was purchased from Formlabs. Syringes were purchased from Becton Dickinson. *Escherichia coli* K12 (ATCC 10798) and *Enterococcus faecalis* (ATCC 19433) were purchased from ATCC. *Salmonella typhimurium* was provided by Dr. Igor Brodsky's lab at the University of Pennsylvania. Heat-inactivated SARS-CoV-2 (NR-52286) and quantitative PCR (qPCR) control RNA from heat-inactivated SARS-CoV-2 (NR-52347) were obtained from BEI Resources. All chemicals used were analytical reagent grade or better. The sequences of the primers used in this study are listed in Tables S1, S2 and S3 [26–30].

2.2. Bacterial cultivation

Escherichia coli K12, *Enterococcus faecalis* and *Salmonella typhimurium* were used as model human enteric pathogens to evaluate the performance of our developed assay. One single colony of *Escherichia coli* K12 or *Salmonella typhimurium* from LB agar plate was incubated in 10 mL of LB broth medium at 37 °C with shaking at 200 revolutions per minute to an OD_{600} of 0.8−1.0. *Enterococcus faecalis* was cultured in the same way using BHI medium. Bacterial cells were washed and resuspended in 0.1 M PBS (pH 7.0) before detection.

### 2.3. Amplification detection of target nucleic acids

The bacterial DNA was first extracted by using the QIAamp DNeasy Blood and Tissue kit. Colorimetric RPA reactions were carried out at 40 °C with 0.48 μM forward (F3) and reverse primer (B3), 14 mM $MgSO_4$, and 120 μM EBT using the TwistAmp Basic reagent. The SEC-LAMP reaction was carried out with 1.6 μM FIP/BIP, 0.2 μM F3/B3, 0.4 μM LoopF/B, 0.32 U/μL *Bst* 2.0 DNA polymerase, 1.4 mM dNTPs, 8 mM $MgSO_4$, and 120 μM EBT in non-buffered LAMP reaction solution [31]. For the colorimetric RAMP reaction [32], RPA was first carried out for pre-amplification at 40 °C for 15 min. Then 1 μL RPA amplicon was added to 14 μL of the non-buffered LAMP solution and incubated at 63 °C for 30 min to complete detection. For detecting SARS-CoV-2 RNA using the RT-RAMP reaction, 2 U AMV reverse transcriptase (Invitrogen, Carlsbad, CA) was added to the RPA reaction solution.

Quantitative PCR (qPCR) was applied to detect *Escherichia coli* K12, *Enterococcus faecalis* and *Salmonella typhimurium*. The qPCR reaction conditions were as follows: 1) *Escherichia coli* K12: 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s; [27] 2) *Enterococcus faecalis*: 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, and 72 °C for 60 s; [28] 3) *Salmonella typhimurium*: 95 °C for 1 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s [30]. The SARS-CoV-2 was detected by RT-qPCR according to the protocol developed by the US Centers for Disease Control and Prevention (CDC). All RPA, LAMP, RAMP, and qPCR reactions in tubes were carried out using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). For SARS-CoV-2, reverse transcription was carried out at 50 °C for 5 min. The initial denaturation at 95/94 °C prior to PCR cycling was intended to fully denature the DNA.

To compare with colorimetric SEC-LAMP detection, traditional fluorescence detection was carried out on microfluidic chips using EvaGreen dye. The fluorescence signal of the LAMP reactors was monitored in real-time using a USB fluorescence microscope (AM4113T-GFBW Dino-Lite Premier, AnMo Electronics, Taipei, Taiwan) [33]. Using MATLAB, the fluorescence intensities of each reactor were extracted, producing normalized average fluorescence intensities for each specified time interval (e.g., 1 min interval).

2.4. FTA-based SMCD

In the FTA-based multiplexed colorimetric assay, the pathogens spiked in water samples were first isolated and concentrated from 30 mL water samples using an FTA membrane at a diameter of 2 mm. After heating the FTA membrane at 40 °C for 15 min, the nucleic acids were released and bound to the FTA membrane. Next, the nucleic acids were purified by the FTA purification reagent to remove potential inhibitors. Then, the FTA membrane with nucleic acids was directly placed into the RPA reaction solution with specific F3/B3 primers and incubated at 40 °C for 15 min (2 U of AMV reverse transcriptase was included for RT-RPA). Lastly, 1 μL the RPA amplicon was added to 14 μL SEC-LAMP reaction solution in separate vials containing specific LAMP primers and incubated at 63 °C for another 30 min for colorimetric SEC-LAMP detection.
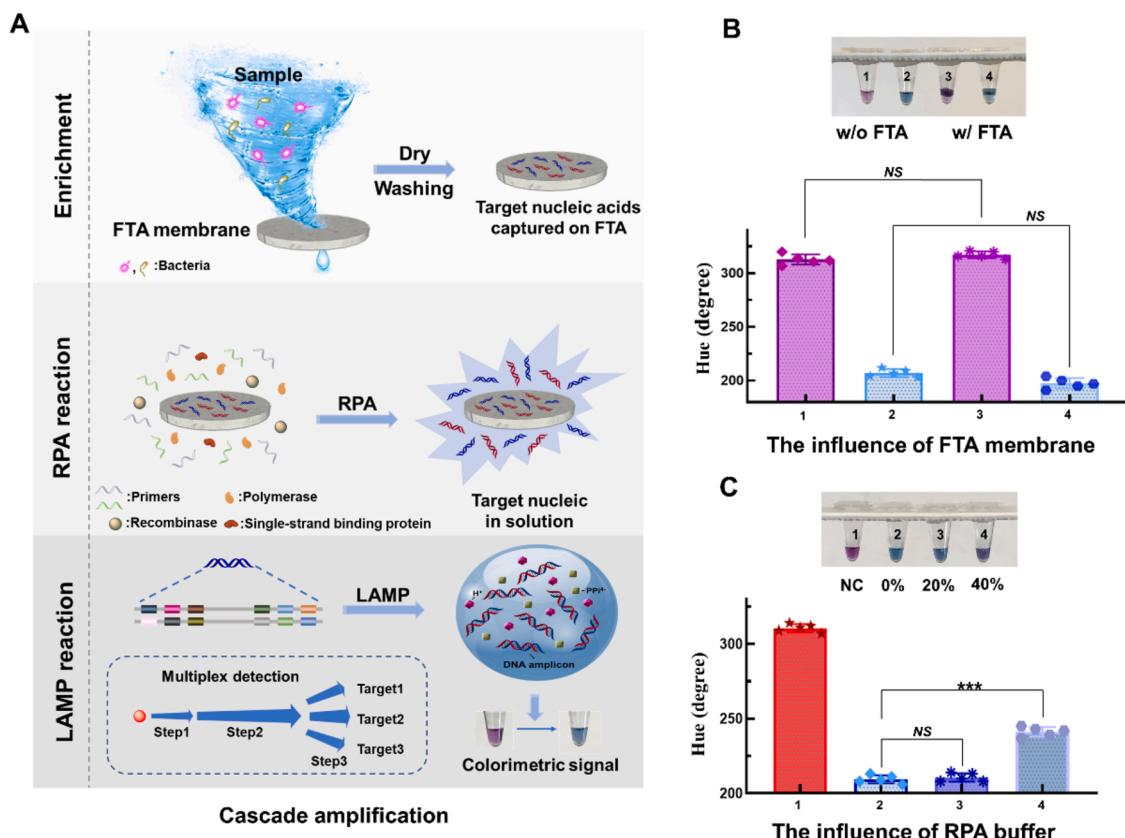
Fig. 1. Design and optimization of FTA-based multiplexed colorimetric assay for the sensitive detection of pathogens in wastewater. (A) Design of FTA-based multiplexed colorimetric assay for the detection of different pathogens. (B) Effect of FTA membrane on the SEC-LAMP assay. Tubes 1-4: negative control without FTA membrane, positive control (500 CFU/reaction, *Salmonella*) without FTA membrane, negative control with FTA membrane, and positive control (500 CFU/reaction, *Salmonella*) with FTA membrane, respectively. (C) Effect of RPA reaction solution volume on the SEC-LAMP assay. Tubes 1-4: negative control, positive control (500 CFU/reaction, *Salmonella*), positive control with 20 % (v/v) RPA reaction solution, and positive control with 40 % (v/v) RPA reaction solution, respectively. NS: no significant difference (p > 0.05, t-test). *** indicates a significant difference (p < 0.001, t-test). Error bars denote s.d. (n = 5).

## 2.5. Integrated microfluidic chip and portable detection platform

The dimensions of the integrated microfluidic chip were 35 mm (L) X 20 mm (W) X 4 mm (H). The chip consisted of two reactor units: i) a multilayer RPA reactor unit integrated with the FTA membrane for nucleic acid enrichment/extraction and RPA pre-amplification of multiple targets, and ii) an SEC-LAMP reactor array unit for multiplexed colorimetric detection. To fabricate the integrated microfluidic chip, it was first designed by using SolidWorks software and fabricated using a Form 2 3D printer (Formlabs) with clear methacrylate-based resin (FormsLabs, FLGPCL02). Next, the printed chips were washed by isopropanol (IPA) and ddH2O for 15 min under ultrasonication conditions to remove the uncured resin. PCR Sealers tape (Microseal® 'B' Film) (Bio-Rad) was used to cover the top and bottom of the microfluidic chip to prevent liquid evaporation. To improve the biocompatibility of the 3D-printed chip, the reactor surface of the chips was modified by either PVA or liquid acrylics [33].

The multiplexed colorimetric assay on the microfluidic chip was carried on a battery-powered portable detection platform for isothermal amplification. As shown in Fig. S1, the portable detection platform was fabricated by 3D printing. A flexible, polyimide-based, thin film heater (Model HK5572R7.5L23A, Minco Products, Inc., Minneapolis, MN) controlled by our custom-made electronic circuit board provided a stable reaction temperature, which was pre-set by MATLAB and pre-calibrated by an Omega Engineering Thermometer HH806AW. The colorimetric signal was recorded in real-time and analyzed by a programmed smartphone with our custom App. As shown in Fig. S2, our custom-developed app can simultaneously detect multiple LAMP chambers, enabling a multiplexed colorimetric assay. The images were captured using the phone camera and the hue value change of each LAMP chamber was extracted, analyzed in real-time, and reported by the smartphone to a custom website.

2.6. Pathogen detection on the integrated microfluidic chip

The disposable 3D printed microfluidic chip contained one RPA reactor with an FTA membrane and four separate SEC-LAMP reactors. To realize on-chip multiplexed colorimetric detection, specific LAMP primers were pre-stored in three separate SEC-LAMP reactors. The fourth SEC-LAMP reactor did not have any primers and served as the negative control. To concentrate target nucleic acids, the water samples were firstly introduced into the microfluidic chip using a 60-mL syringe and flowed through the FTA membrane on the multilayer RPA reactor. After dried at 40 °C for 15 min on the portable detection platform, the nucleic acids were released on the FTA membrane and purified with 100 μL FTA purification reagent. Next, the lid was placed on the RPA reactor and the outlet of RPA reactor was sealed with PCR tape (Microseal® 'B' Film). Next, 10 μL RPA reaction solution with F3/B3 primer mixture was introduced into the RPA reactor and incubated at 40 °C on the portable detection platform for 15 min. At the end of the RPA reaction, the outlet (outlet 2) of the LAMP reaction chambers was pre-covered with a hydrophobic porous membrane, which obstructed liquid reagent but not air, and ensured that all four reaction chambers were fully filled without voids. Then, 100 μL non-buffered LAMP solution was added to the RPA reactor, mixed with the RPA amplicons and split into four separate SEC-LAMP reactors. After the chip was sealed, it was incubated at 63 °C on
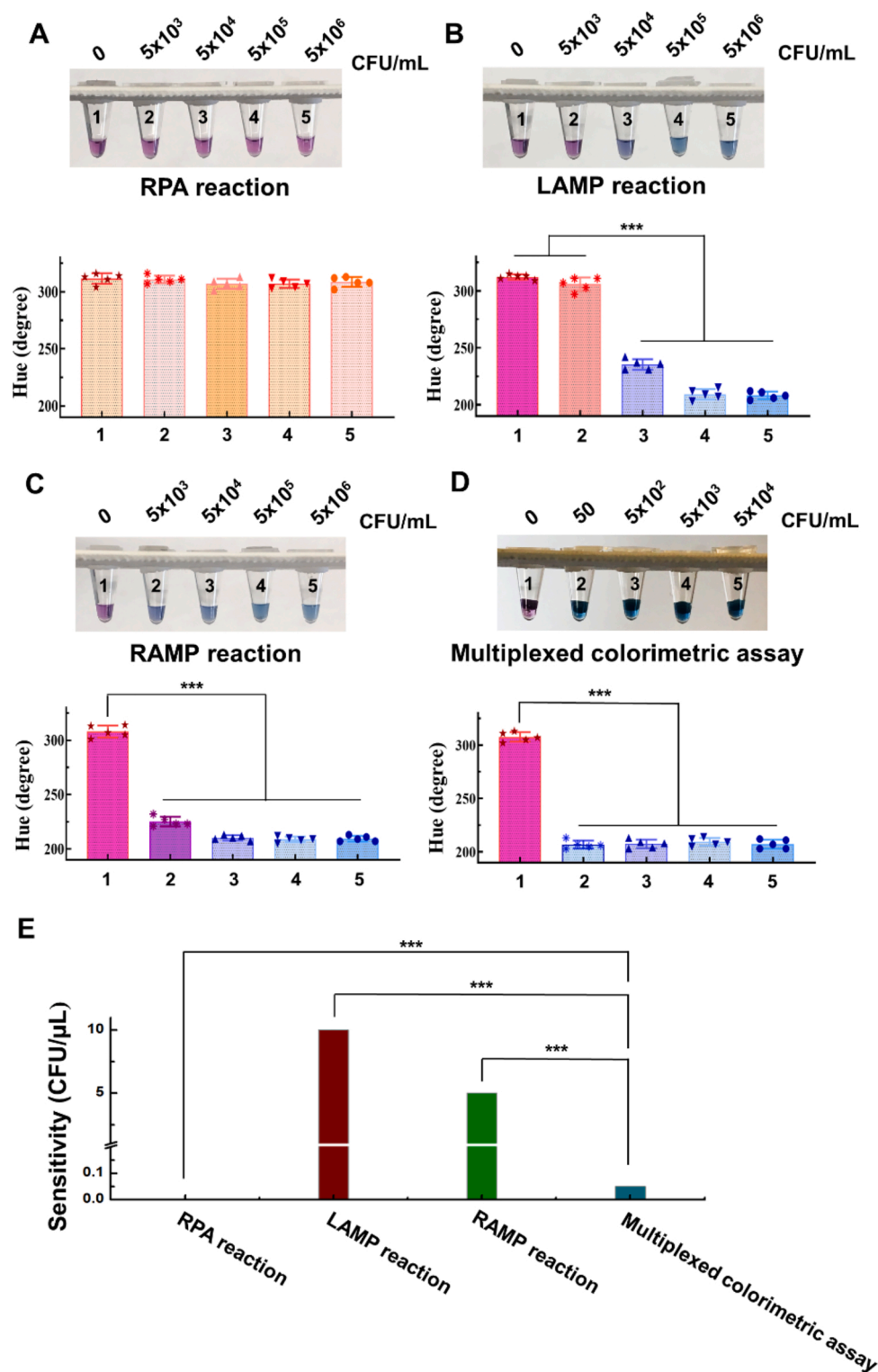
Fig. 2. Sensitivity comparison of the FTA-based multiplexed colorimetric assay with other methods. (A-D): Detection of 10-fold serial dilutions of *Salmonella* DNA using RPA, LAMP, RAMP, and FTA-based multiplexed colorimetric assays, respectively. (E) Sensitivity comparison of different detection methods.

the portable detection platform for the colorimetric SEC-LAMP assay. The colorimetric signal was recorded in real-time and analyzed by our programmed smartphone with a custom App on the portable detection platform.

2.7. Water sample analysis

The water samples were collected from Farmington River in CT, USA. The collected river water samples were directly detected on-site on the 3D printed microfluidic chip with our smartphone-based detection platform. Test results were reported and wirelessly transmitted to our custom website, enabling pathogen tracking and water quality
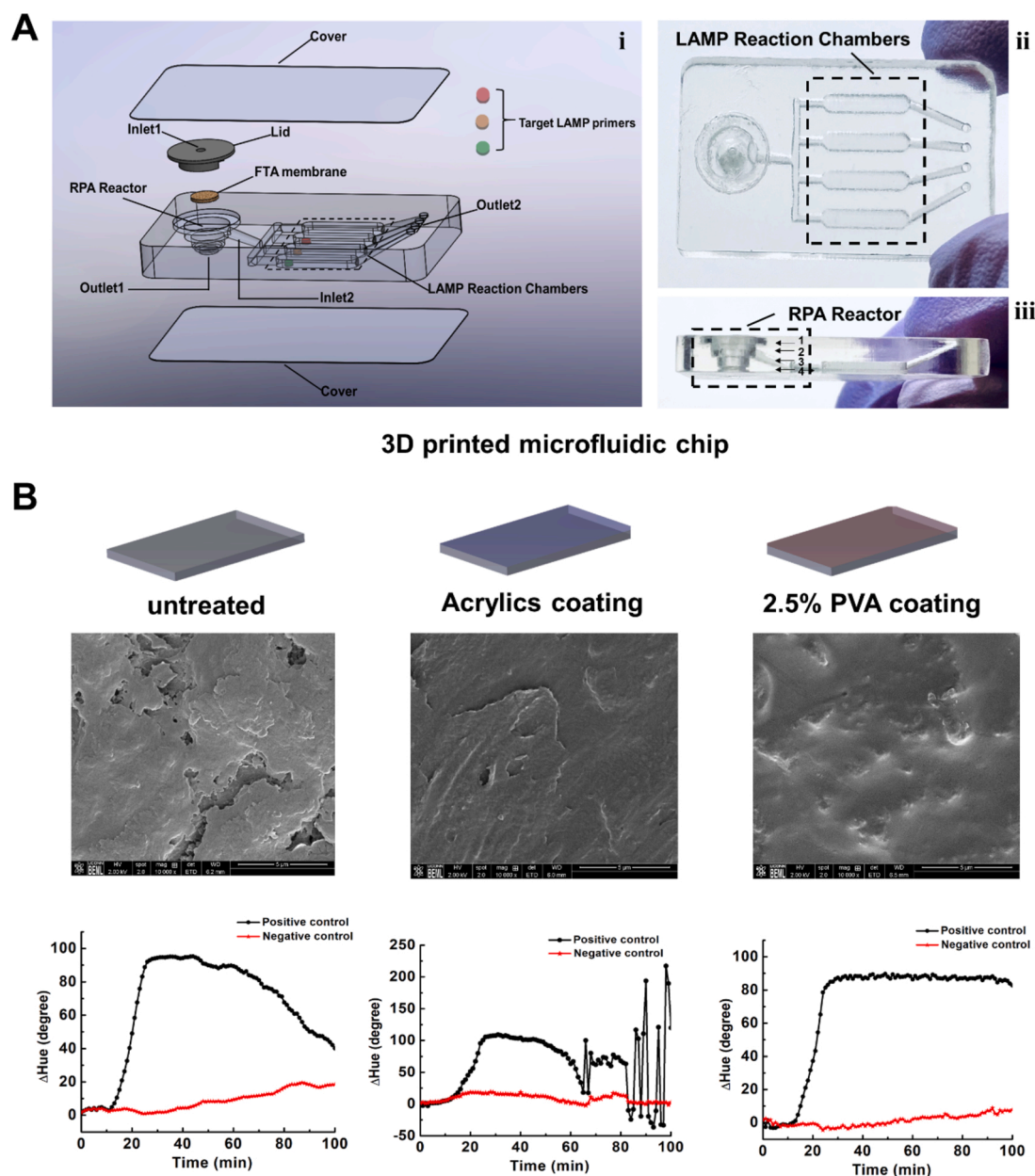
Fig. 3. Integrated microfluidic chip for the sensitive, multiplexed, colorimetric assay. (A) Microfluidic chip. i: exploded view; ii: top view; iii: side view. (B) Surface modification of the microfluidic chip with acrylics coating and hydrophilic PVA coating. Surface images were taken by a scanning electron microscope. Real-time ΔHue curves of positive and negative controls were collected on the portable detection platform.

surveillance. To further evaluate the performance of our method, the collected river water samples were brought to our laboratory and spiked with different pathogens for testing.

Municipal wastewater samples (raw influent, 50 mL) were collected at 2:30 pm each day for 10 days at the University of Connecticut, Water Pollution Control Facility in Storrs, CT, USA. The typical characteristics of the raw wastewater samples, such as chemical oxygen demand (COD), total suspended solid (TSS), ammonium, alkalinity, and conductivity were measured using standard methods on the same day of sampling. The wastewater samples were filtered using Whatman filter before detection. Since only *Enterococcus faecalis* was detected from our collected wastewater samples, SARS-CoV-2, *Escherichia coli K12*, and *Salmonella typhimurium* were spiked in the wastewater samples to further evaluate the detection performance of our SMCD method in the laboratory.

3. Results and discussion

3.1. Design and establishment of FTA-based SMCD

On-site, sensitive and multiplexed detection of pathogens in wastewater remains a challenge due to the methodological limitations. To address this challenge, a simple, rapid and reliable method is needed to enrich pathogens and extract nucleic acids from water samples. In recent decades, the development of FTA membrane has enabled a simple method for collecting, shipping and purifying nucleic acids [34–36]. In this study, using the FTA membrane to enrich pathogens, lyse cells, capture and purify nucleic acids from wastewater samples, we developed a simple, FTA-based SMCD method (Fig. 1A) to simultaneously detect multiple pathogens from wastewater samples by combining FTA-based nucleic acid sample preparation, RPA pre-amplification and SEC-LAMP detection.
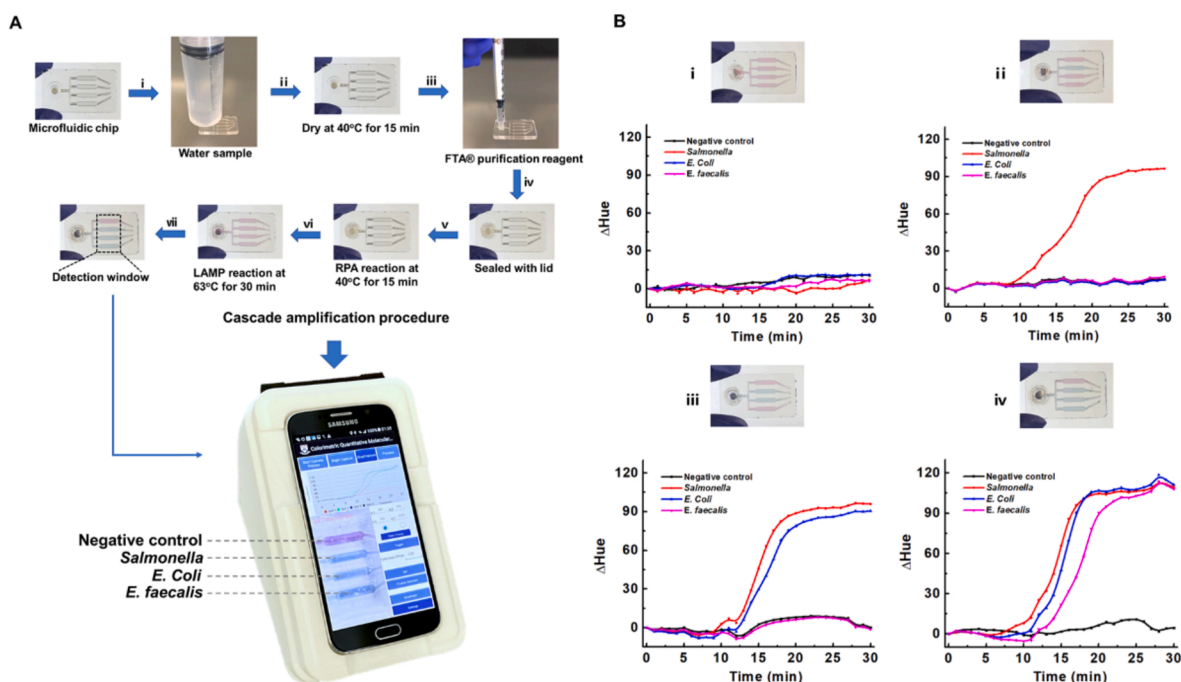
Fig. 4. Multiplexed detection of human enteric pathogens on our 3D printed microfluidic chip. (A) Procedure of multiplexed colorimetric detection. (B) Multiplexed pathogen detection in water samples: i: ddH$_2$O (negative control); ii: ddH$_2$O spiked with 500 CFU/mL *Salmonella*; iii: ddH$_2$O spiked with 500 CFU/mL *Salmonella* and *Escherichia coli* K12; iv: ddH$_2$O spiked with 500 CFU/mL *Salmonella*, *Escherichia coli* K12 and *Enterococcus faecalis*.

To investigate the compatibility of the FTA membrane with SEC-LAMP detection, the FTA membrane washed by the FTA purification solution was placed into non-buffered LAMP solution for *Salmonella* DNA detection. As shown in Fig. 1B, the presence of the FTA membrane did not influence the SEC-LAMP detection. After nucleic acids captured by the FTA membrane, multiplex RPA reactions were designed to further amplify target nucleic acids by taking advantage of its speed, high sensitivity and low operation temperature (25−42 °C) [37]. In addition, a previous study demonstrated that the RPA reaction is compatible with conventional fluorescence LAMP detection in the RAMP assay [32]. In our previous study [31], we demonstrated that the SEC-LAMP assay with a non-buffered solution can significantly improve the colorimetric readout and detection sensitivity. However, the TwistAmp Basic reagent for the RPA reaction itself is a buffered reaction system. To investigate the influence of RPA reaction solution on the SEC-LAMP detection, we evaluated various volumes of the RPA reaction solution in the SEC-LAMP detection. As shown in Fig. 1C, no significant inhibition effect arose when less than 20 % (v/v) RPA reaction solution was added to the SEC-LAMP detection. Hence, combining FTA-based target nucleic acid enrichment/extraction, RPA pre-amplification and SEC-LAMP detection, we developed a sensitive, colorimetric method for simultaneous detection of various pathogens.

### 3.2. Sensitivity of FTA-based SMCD

To evaluate the sensitivity of FTA-based multiplexed colorimetric assay, we detected 10-fold serial dilutions of *Salmonella* DNA and compared our results with those of other detection methods. As shown in Fig. 2A, no obvious color change was observed in the RPA method, indicating that the proposed colorimetric detection was not suitable for the RPA reaction. As shown in Fig. 2B-C, sensitivities of 1000 and 500 colony-forming units (CFU)/mL were realized by the SEC-LAMP method and RAMP method, respectively. In comparison, we detected *Salmonella* at less than 50 CFU/mL by the FTA-based multiplexed colorimetric assay, due to the cascading amplification effect, including FTA-based nucleic acid enrichment, RPA pre-amplification and SEC-LAMP detection (Fig. 2D-E). Further, we also detected *Enterococcus faecalis* and

Escherichia coli K12 with SMCD with similar sensitivities (Fig. S3). To our best knowledge, this is the first time that the FTA membrane has been utilized to enrich pathogens and extract nucleic acid from large-volume water samples.

Next, we evaluated the quantitative detection ability of our FTA-based multiplexed colorimetric assay with 10-fold serial dilutions of human enteric pathogens in water samples. As shown in Fig. S4, there was a statistically significant difference in threshold time between high-concentration (5×10^4 CFU/mL) and low-concentration groups (500 CFU/mL) for *Salmonella*, *Enterococcus faecalis* and *Escherichia coli* K12. However, because of the separate RPA pre-amplification step, the SMCD method can only provide semiquantitative detection. In addition, we investigated the multiplexed detection ability of our developed method. As shown in Fig. S5, we simultaneously detected various human enteric pathogens in a single water sample with excellent specificity by using the FTA-based multiplexed colorimetric assay. Together, these results confirm that our FTA-based SMCD method is suitable for sensitive and multiplexed detection of human enteric pathogens.

3.3. 3D printed integrated microfluidic chip

Microfluidics technology enables sample manipulation, biochemical reaction and microscale analysis on a miniaturized chip [38]. Recently, 3D printing offers rapid, low-cost, one-step fabrication of complex microfluidic devices for various biomedical applications [24,39]. To simplify sample preparation process, FTA membrane was directly integrated on the bottom layer (the fourth layer) of the RPA reactor for concentrating/lysing pathogens, and extracting/purifying nucleic acids (Fig. 3A). As shown in Fig. 3A-i, and iii, the third layer fits tightly against the syringe tip, which separates the SEC-LAMP reactors from the DNA extraction process because the inlet 2 of the SEC-LAMP reactors is located on the second layer. After RPA reaction, the non-buffered LAMP solution was firstly mixed with RPA amplicons in the RPA reactor and then distributed into four SEC-LAMP reactors (Fig. 3A-ii), enabling sensitive, multiplexed colorimetric detection. As shown in Fig. S6, the lyophilized LAMP primers pre-stored in the microfluidic chip are stable at room temperature for at least 30 days without obvious efficiency
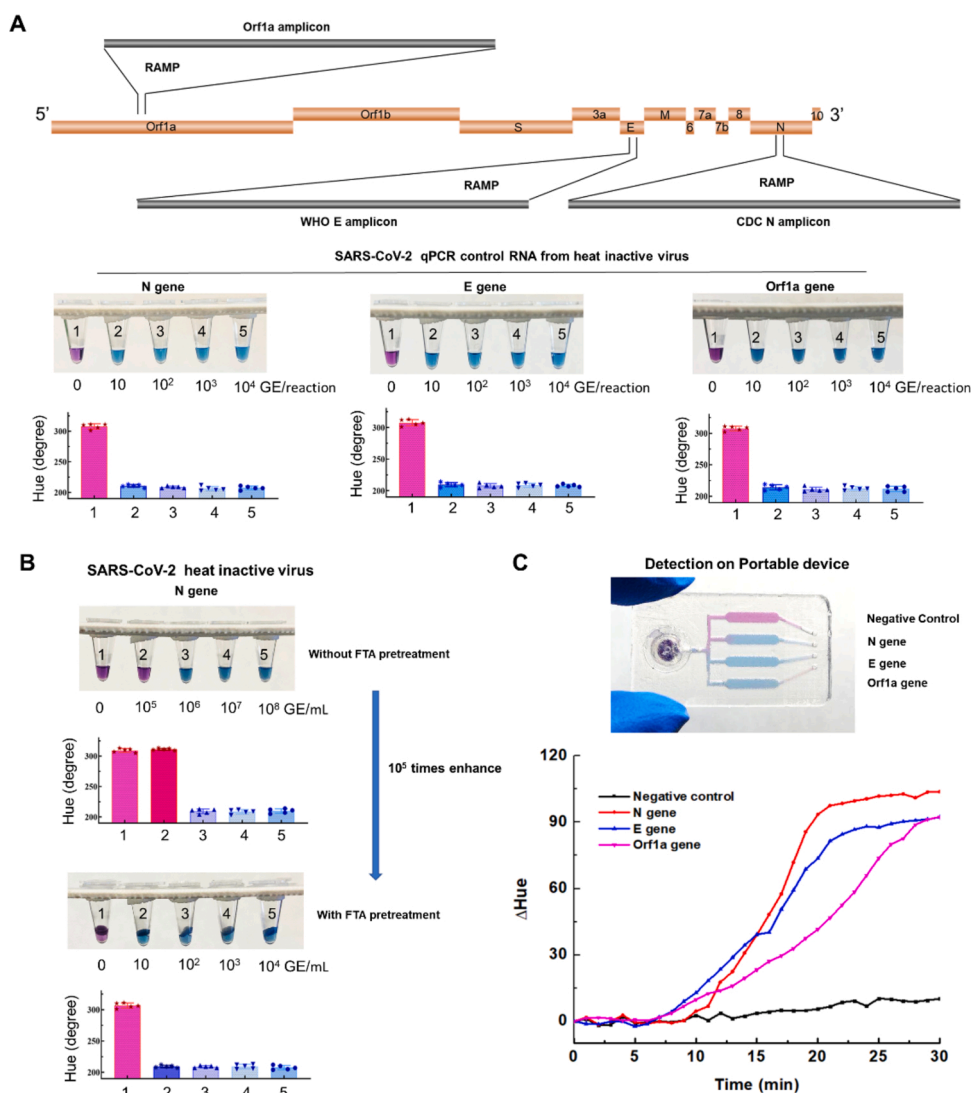
Fig. 5. Multiple gene detection of SARS-CoV-2. (A) Colorimetric detection of the N gene, E gene and Orf1a gene of SARS-CoV-2 RNA by RT-RAMP reactions. (B) Colorimetric detection of the N gene of SARS-CoV-2 by the RT-RAMP reaction with/ without FTA membrane pre-treatment. Error bars denote s.d. (n = 5). (C) Colorimetric detection of the N gene, E gene, and Orf1a gene of SARS-CoV-2 on a 3D printed microfluidic chip.

decrease. If desired, more SEC-LAMP reactors can be designed to simultaneously detect more pathogens. The resin cost for printing a 3D chip is ~ 0.2 $, nucleic acid extraction reagents cost ~ 0.3 $, and assay reagents are ~ 1.5 $, leading to a total cost per assay of ~ 2.0 $.

The clear resin was used to fabricate the 3D printed microfluidic chip due to its excellent transparency and high temperature resistance. To improve the biocompatibility and signal stability, we modified the surface of the reactors with different coating materials, such as acrylics and PVA. As shown in Fig. 3B, both the untreated chip and acrylics-treated chip amplified target nucleic acid, however, the colorimetric signal of the positive groups gradually decreased with increasing reaction time. The decreased colorimetric signal on the untreated chip and acrylics-treated chip may result from the generation of bubbles during isothermal amplification. Among the chips tested, the PVA-treated chip exhibited the most reliable colorimetric signal in the SEC-LAMP assay. Next, we further optimized the PVA concentration by treating the LAMP reaction chambers with various concentrations of PVA (1%, 2.5 %, 5% and 10 %). As shown in Fig. S7, the SEC-LAMP reactors coated with 2.5 % PVA showed the best performance in our assay. Thus, 2.5 % PVA coating was used in the remainder of the experiments.

3.4. Human enteric pathogen detection on the integrated microfluidic chip

To evaluate the multiplexed detection ability of the integrated microfluidic chip, ddH₂O water samples spiked with 500 CFU/mL of different types of bacteria (e.g., *Salmonella*, *Enterococcus faecalis* and *Escherichia coli* K12) was detected (Fig. 4A). As shown in Fig. 4B and Fig. S8, we achieved simple, multiplexed, colorimetric detection of human enteric pathogens on the chip with excellent specificity within one hour. Although the fluorescence detection is widely used for nucleic acid testing, this approach typically requires a complex optical design and expensive optical filers or excitation light sources, which increase the detection cost and are not ideal for on-site applications. Compared to fluorescence detection, our colorimetric detection has distinct advantages, including simplicity, low-cost, and convenience, which is vital for developing the portable detection platform. Our previous study demonstrated that hue value-based colorimetric detection is superior to traditional RGB (red, green and blue)-based colorimetric assays [31]. Therefore, we adapted hue-based colorimetric detection in our smartphone-based detection platform. For comparison, we performed real-time fluorescence detection by using a hand-held microscope and analyzed the results using MATLAB (Table S4) [33]. As shown in Fig. S9, the reaction threshold time of real-time colorimetric detection was
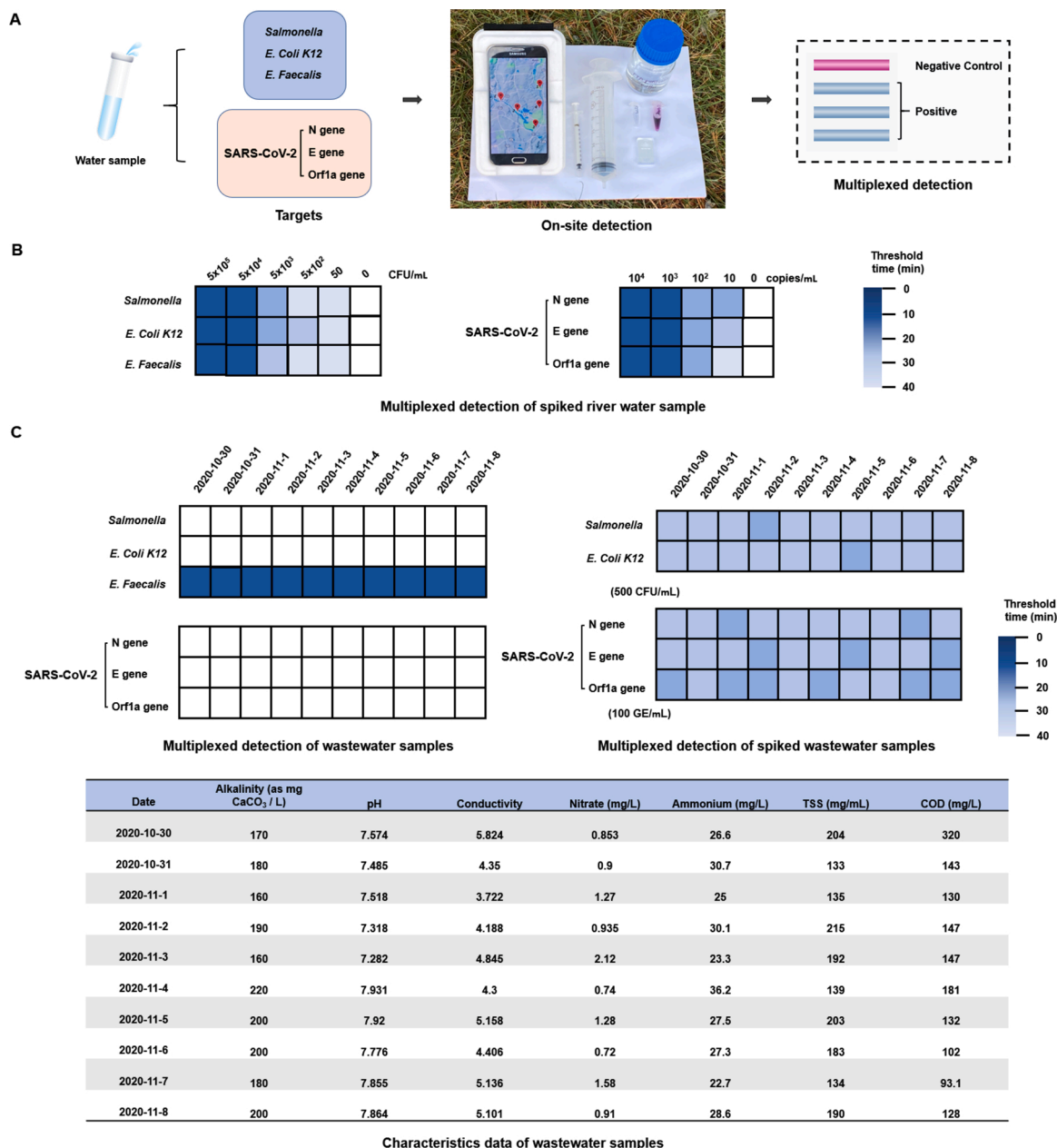
Fig. 6. Detection of SARS-CoV-2 and human enteric pathogens in different water samples by the SMCD method on the portable detection platform. (A) Photograph of the toolbox of our portable detection platform for SARS-CoV-2 and human enteric pathogen detection. (B) Heat map for the detection of serially diluted SARS-CoV-2 and human enteric pathogens in spiked river water samples. (C) Heat map for the detection of SARS-CoV-2 virus and human enteric pathogens in non-spiked and spiked wastewater samples. The table shows the basic characteristics of the wastewater samples.

shorter than that of the fluorescent detection method and had a lower variation, which may result from the synergistic enhancement of Mg²⁺ detection during the SEC-LAMP reaction. Together, the combination of a colorimetric assay with our integrated microfluidic chip provides a simple, rapid, cost-effective method for pathogen detection without the need for complex equipment.

3.5. Detection of multiple genes of SARS-CoV-2 on the integrated microfluidic chip

The outbreak of SARS-CoV-2 has resulted in global pandemic [40]. Several studies have confirmed that the detection of SARS-CoV-2 in wastewater may offer a powerful tool for understanding its epidemiology because wastewater provides an aggregate sample that can be more easily acquired than pooled clinical samples [3,7,41]. Here, we

applied our integrated 3D printed microfluidic chip to simultaneously detect multiple target genes of SARS-CoV-2, including the E (envelope), N (nucleoprotein), and ORF1a genes [26,42]. As shown in Fig. 5A, we consistently detected the N gene, E gene and Orf1a gene from purified SARS-CoV-2 RNA with a sensitivity of 10 genome equivalents (GE)/reaction with the RT-RAMP assay. For SARS-CoV-2 virus detection, we achieved a sensitivity of only 10⁶ GE/mL by the RT-RAMP assay without any sample pretreatment (Fig. 5B). However, with the pretreatment by FTA membrane, we detected 10 GE/mL SARS-CoV-2 virus in a ddH₂O sample, which may result from the highly efficient pathogen enrichment and lysing ability of the FTA membrane (Fig. 5B). Next, we detected the multiple genes of SARS-CoV-2 on 3D printed microfluidic chip. As shown in Fig. 5C, we detected all three target genes of SARS-CoV-2 with a sensitivity of 10 GE/mL in ddH₂O on the integrated chip.

3.6. Pathogen detection in river water and wastewater samples

Rapid detection of pathogens in water samples is vital for assessing water quality and can be used as an early warning tool for monitoring disease outbreaks. To demonstrate the feasibility of the SMCD method to directly detect SARS-CoV-2 and human enteric pathogens in water samples in the field, we collected river water samples from Farmington River (CT, USA) at different locations and tested them on our platform. Fig. 6A shows the toolbox of our portable microfluidic detection platform for SARS-CoV-2 and human enteric pathogen detection, which consists of: i) a portable detection platform with a programmed smartphone, ii) 3D printed, integrated microfluidic chips, iii) disposable syringes, and iv) reagent tubes. By taking advantage of the international connectivity and global position system (GPS) tracking of our smartphone-based detection platform, the detection information (e.g., test results, pathogen types, location, test time) could be reported on our custom-website, enabling smart and connected pathogen detection and spatial mapping (Fig. 6A). Because negative results were obtained for the river water samples, the collected river water samples were taken back to the laboratory and spiked with serial-diluted pathogens. Next, these water samples were detected by the multiplexed colorimetric assay on the integrated microfluidic chip. As shown in Fig. 6B, our platform detected SARS-CoV-2 virus with a sensitivity of 10 GE/mL within one hour. The platform also achieved sensitivities of 50 CFU/mL for human enteric pathogens, which was comparable to that of the qPCR methods [20,43].

Previous studies have reported that the concentration of SARS-CoV-2 in wastewater environments (e.g., wastewater, sludge) ranges from 1.0×10^3 to 4.6×10^5 copies/mL [9,44]. The concentrations of human enteric pathogens (e.g., *Enterococcus faecalis*, *Escherichia coli*) in the wastewater vary from 10 CFU/mL to 10^4 CFU/mL [45–47]. To further investigate the performance of our SMCD method, raw wastewater samples were collected from the influent point of the UConn Water Pollution Control Facility over a 10-day sampling period (10/30/2020-11/8/2020). The raw wastewater quality exhibited variations, with ranges of 93−320 mg/L for the COD, 133–215 mg/L for the TTS, 4.3–5.8 μS/cm for conductivity, 23−36 mg/L for ammonium, 160−220 mg/L for alkalinity as $CaCO_3$, and ~ 7.3–7.9 for the pH (relatively stable). As shown in Fig. 6C, *Enterococcus faecalis* was directly detected in the wastewater samples, which was confirmed by the PCR method (Fig. S10, S11). To further evaluate the detection performance, we also spiked inactivated SARS-CoV-2 virus, *Escherichia coli K12*, and *Salmonella typhimurium* in the wastewater samples in our laboratory. For comparison, qPCR/RT-qPCR methods were also run in parallel (Fig. S10). As shown in Fig. S12, we could detect 100 GE/mL of SARS-CoV-2 virus and 500 CFU/mL of human enteric pathogens (e.g., *Escherichia coli K12*, and *Salmonella typhimurium*) in wastewater, which was comparable to the qPCR/RT-qPCR assay (Table S5 and S6). We note that the detection sensitivity in the wastewater samples is lower than that of the river water by a factor of 10, which may be attributed to the existence of potential inhibitors in the wastewater samples. Additionally, the detection time of our SMCD method was about 1 h, which is half the time required for the RT-qPCR/qPCR method (Fig. S13 and Table S6). Because the flow-through FTA membrane in our SMCD method decouples the sample volume to enrich pathogens, the sensitivity can be further improved by increasing the sample volume and FTA membrane size. Together, the above results demonstrate that our platform provides a simple, rapid, and sensitive method for on-site detection of SARS-CoV-2 and human enteric pathogens in water samples, presenting great potential for disease outbreak surveillance in contaminated water.

4. Conclusions

In summary, we developed a simple, sensitive, multiplexed, colorimetric assay for rapid detection of SARS-CoV-2 and human enteric pathogens in wastewater using an integrated 3D printed microfluidic chip. Compared with conventional fluorescence detection method, the colorimetric signals of our method could be either read by the naked eye or analyzed by the smartphone, eliminating need for complicated equipment and enabling on-site simultaneous detection of SARS-CoV-2 and human enteric pathogens from real river water samples and wastewater samples. By combining RPA and SEC-LAMP assay, the developed SMCD method achieved the multiplexed and sensitive detection with sensitivities of 100 GE/mL and 500 CFU/mL for SARS-CoV-2 and human enteric pathogens in wastewater, respectively. Further, we have demonstrated smart and connected detection of waterborne pathogens in the field, which have great potentials in water quality monitoring and contamination sources tracking.

CRediT authorship contribution statement

Kun Yin: Conceptualization; Methodology; Investigation, Data collection; Validation; Writing – original draft, Reviewing & Editing. **Xiong Ding**: Data collection; Formal analysis; Validation; Writing –Reviewing & Editing. **Zhiheng Xu**: Data collection; Formal analysis; Writing –Reviewing & Editing. **Ziyue Li**: Data collection; Formal analysis; Writing –Reviewing & Editing. **Xingyu Wang**: Sample Collection; Data collection; Formal analysis; Writing –Reviewing & Editing. **Hui Zhao**: Funding acquisition; Formal analysis; Writing –Reviewing & Editing. **Clifford Otis**: Sample Collection; Data collection; Writing –Reviewing & Editing. **Baikun Li**: Sample Collection; Resources; Formal analysis; Validation; Writing –Reviewing & Editing. **Changchun Liu**: Conceptualization; Methodology; Funding acquisition; Project administration; Supervision; Writing – original draft, Reviewing & Editing

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2021.130242.

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