ACCELERATE SEPSIS DIAGNOSIS BY SEAMLESS INTEGRATION OF DNA PURIFICATION AND QPCR

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ABSTRACT

The high mortality rate of sepsis has been attributed to delayed pathogen identification. Hence, a fluidic device is being developed to accelerate the procedures from DNA purification to qPCR sequence detection. Demonstrated herein is the key feature the device: the use of EWD droplet actuation to manipulate the eluent generated by an immiscible phase filtration DNA preprocessor. Among other benefits, EWD actuation enables the eluent to be compartmentalized into several microdrops for subsequent automated PCR mixture preparation and multiplex sequence detection. Also discussed are the characterization of purification power, and the fabrication of the high temperature tolerance plastic top plate.

KEYWORDS

Digital microfluidics, immiscible phase filtration, nucleic acid purification, sample preparation, PCR inhibitor

INTRODUCTION

Septic shock is a severe form of bloodstream infections. Without effective antimicrobial therapy, mortality rate reaches 58% within five hours after the onset of hypotension [1]. In contrast, SeptiFast, a commercial multiplex sparse pathogen detection assay involving DNA purification followed by qPCR sequence detection, requires three hours of operator time and six hours of total run time [2].

To address the above discrepancy, a temperature zone PCR device augmented with a DNA preprocessor is being developed. The preprocessor utilizes the high interfacial tension between a lysate and an immiscible phase to minimize the carryover of PCR inhibitors when DNA-bound magnetic silica beads are snapped from a lysate (Fig. 1a) [3]. Next, by dispersing the beads in wash buffers, PCR inhibitors in the carryover are digested or diluted. Later, after DNA is released from the beads to the elution buffer, DNA-containing eluent microdrops are generated and then mixed with PCR reagents using electrowetting-on-dielectric (EWD) actuation. Afterwards, rather than thermocycling the entire device, PCR can be executed by shuttling the resulting microdrops between 60 °C and 95 °C zones (Fig. 2a) [4]. Compared with benchtop assays, this approach aims to (a) reduce operator involvement by automating DNA purification and PCR mixture preparation, and (b) minimize PCR time by maximizing the temperature ramping rate.

Although a similar concept has been demonstrated [4], its droplet actuation mechanism requires magnetic beads to remain in a single droplet. Thus, the reported device is limited to performing a single PCR reaction. Further, the presence of beads in PCR mixtures is observed to reduce the amplification efficiency by 50% [5]. We assert that these two flaws are solved in this study by the use of EWD droplet actuation in the qPCR subsystem.

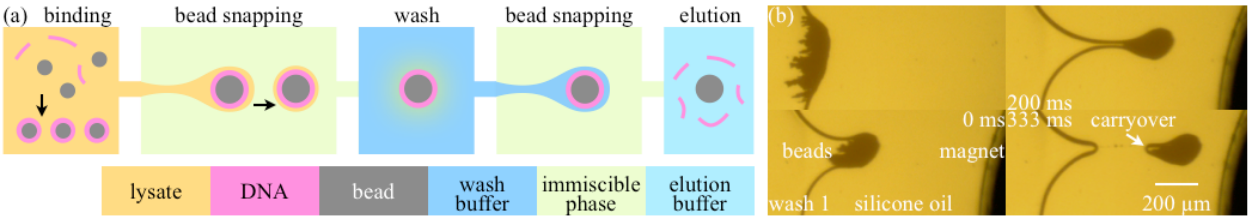

Figure 1. (a) Schematic of an immiscible phase filtration DNA preprocessor, top-down view. DNA is purified from a lysate as magnetic beads are transported through the preprocessor. (b) The sequence of micrographs shows that after wash 1 the beads were snapped from wash buffer 1 to the immiscible phase.

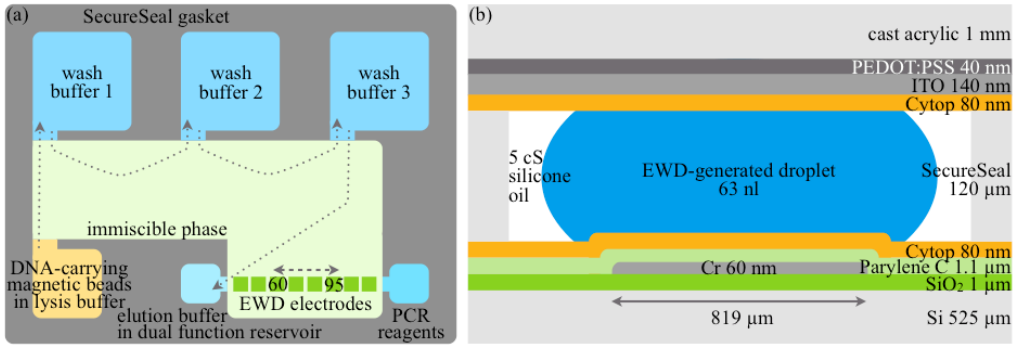

Figure 2. (a) Floor plan. Dotted line: DNA-carrying beads are transported through the preprocessor by an external magnet. Dashed line: microdrops are transported by EWD actuation. To perform PCR, an eluent microdrop mixed with PCR reagents is shuttled between the two temperature zones. (b) Device structure.

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EXPERIMENTAL

Device: The structure and the floor plan of the device are shown in Fig. 2. There are two improvements over typical digital microfluidic devices. First, compared with SU-8 gaskets, the laser-patterned SecureSeal gasket halves the fabrication time and provides an optimal confinement of reagents in their respective reservoirs.

Second, the ITO/PEDOT:PSS/acrylic top plate has been tested to withstand > 100 °C, which is sufficient for the 95 °C denaturing step in PCR. In contrast, since the mismatch of thermal expansion coefficients causes the ITO grounding electrode to crack, an ITO/acrylic top plate can only withstand < 60 °C. Although PEDOT:PSS does not prevent the ITO from cracking, after 100 °C baking the sheet resistance remains unchanged at 3 ohm/sq. Further, after baking the assembled devices that are loaded with the reagents at 95 °C, EWD droplet actuation is observed to function normally. Notably, oxygen plasma ashing of the acrylic immediately prior to the spin coating of PEDOT:PSS is found to be essential to the enhancement of temperature tolerance. The behavior might be related to the hydrophilicity of PEDOT:PSS. Lastly, PEDOT:PSS/acrylic and ITO/glass top plates are not currently employed because for the former, it seems preferable to avoid the contact of water-soluble PEDOT:PSS with aqueous reagents; for the latter, it is difficult to pattern the pipette ports on glass substrates.

Operation: (1) *DNA binding, reagent loading:* Following a modified NucliSENS miniMAG protocol, 160 bp synthetic DNA or HeLa gDNA is bound to the magnetic silica beads. Then, via the pipette ports on the top plate, the following reagents are loaded into their respective reservoirs: 4 µl beads suspended in the lysis buffer, 8 µl wash buffer 1 ~ 3, 1 µl elution buffer, and 5 cS silicone oil.

(2) *Immiscible phase filtration, wash:* Actuated by an external magnet, the beads are snapped out of the lysis buffer to the immiscible phase. During snapping, the bead pellet is encapsulated by the carryover (Fig. 1b.) Because it is energetically unfavorable for aqueous contaminants to enter the immiscible phase, the volume of the carryover is limited. In this simplified case, the primary PCR inhibitor in the carryover is the GuSCN chaotropic salt that exists in the lysis buffer and wash buffer 1 (Fig. 4a.) Later, by dispersing the beads in the three wash buffers, PCR inhibitors in the carryover are digested or diluted.

(3) *Elution:* After the three washes, DNA-carrying beads are transported to the dual function reservoir. Here, because of the low ionic strength, DNA is released from the beads to the elution buffer. After the 60 °C 5 min elution step, the beads are transported away from the dual function reservoir to prevent the beads from lowering the PCR amplification efficiency. In particular, the dual function reservoir is the interface between the DNA preprocessor and the qPCR subsystem. Specifically, it is the last stage of the DNA preprocessor (stores the elution buffer) and the first stage of the qPCR subsystem (compartmentalizes the eluent into microdrops.)

(4) *Analysis:* After elution, eluent microdrops can be dispensed from the dual function reservoir using EWD actuation for subsequent on-chip qPCR. Additionally, the droplets can be pipetted out of the fluidic device for offline qPCR, PicoGreen dsDNA quantification, and NanoDrop absorbance measurements.

RESULTS AND DISCUSSION

Purification power: During standard benchtop purification, PCR inhibitors are typically diluted by milliliters of wash buffers. In contrast, to lower the concentrations of PCR inhibitors in eluent droplets, the DNA preprocessor in our work primarily relies on minimizing the carryover volume. To evaluate the effectiveness of different purification methods, their purification powers (PP) can be compared.

Defined as (inhibitor concentration inputted to preprocessor) / (inhibitor concentration outputted by preprocessor), PP can be estimated by taking advantage of the GuSCN that already exists in the lysis buffer and wash buffer 1. Between the two buffers, wash buffer 1 is encountered later in the purification process, and at 5 M its GuSCN is relatively concentrated. Hence, $C_{inhibitor,in}$ = 5 M is assumed. Further, qPCR is determined to suffer from delayed threshold cycles at GuSCN > 25 mM in reaction mixtures. Also, the dilution incurred by offline qPCR in this case is 5x. Thus, because qPCR of the eluent generated by the preprocessor does not feature delayed threshold cycles, $C_{inhibitor,out}$ might be conservatively approximated at 25 mM x 5 = 125 mM. Therefore, PP = 5 M / 125 mM = 40.

Note that, because of the constraint of the employed technique (GuSCN < 25 mM does not further lower the threshold cycle), 40x represents an estimated lower bound of PP. As a comparison, simulated purifications conducted on preprocessors of a different design indicate that an optimized system (DNA preprocessor and purification chemistry) can deliver a PP of 10^2 - 10^3x per wash.

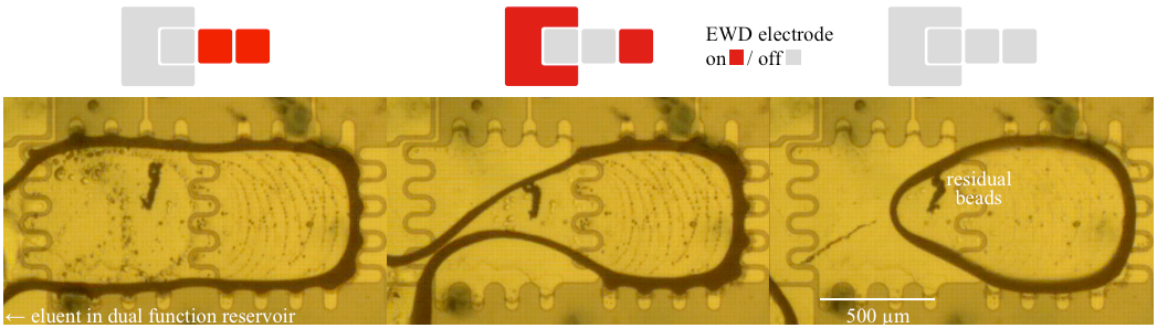

Figure 3. In this series of micrographs, an eluent droplet is dispensed from the dual function reservoir with a minimum amount of residual beads.

DNA preprocessor, EWD subsystem integration: After elution, qPCR sequence detection can be initiated by mixing each eluent droplet generated from the dual function reservoir with a different primers/probe droplet. Hence, in addition to having several sets of primers/probe in a droplet, multiplex detection can also be achieved by performing qPCR concurrently on several droplets.

In contrast, in a previous platform that involves a similar preprocessor, the reaction mixture undergoing temperature zone PCR is actuated via the magnetic beads that remain in the eluent after elution [4]. Yet, the presence of beads in reaction mixtures has been reported to reduce the amplification efficiency by 50% [5]. More importantly, since the platform is incapable of compartmentalizing the eluent into droplets, it seems that the entire eluent has to be subjected to the same PCR reaction. Consequently, the degree of multiplexing that could be attained is limited.

Highlighted in the above discussion is a key feature of our purification-to-quantification device: the superior parallel detection capability provided by using EWD actuation to manipulate the eluent (compartmentalize, transport, and then mix with PCR reagents) generated by the DNA preprocessor. Here, multiplexing is indispensable because there are > 20 pathogens that can lead to sepsis [2].

To verify the feasibility of the outlined approach, the integration of the DNA preprocessor with a downstream EWD subsystem has been demonstrated. Specifically, it is shown that: (1) After on-chip purification, eluent droplets can be dispensed from the dual function reservoir with a minimum amount of residual beads (Fig. 3.) (2) Relative to the concentration before binding, dsDNA in the eluent droplets is 13x more concentrated (Fig. 4b.) This enrichment of DNA is accomplished by minimizing the eluent volume. Crucially, the enrichment improves the detection limit, which is vital to the sensing of sparse pathogens. Lastly, the demonstration also showcased the idea of utilizing a reservoir as a bidirectional bridge between monolithically integrated EWD and non-EWD based fluidic subsystems.

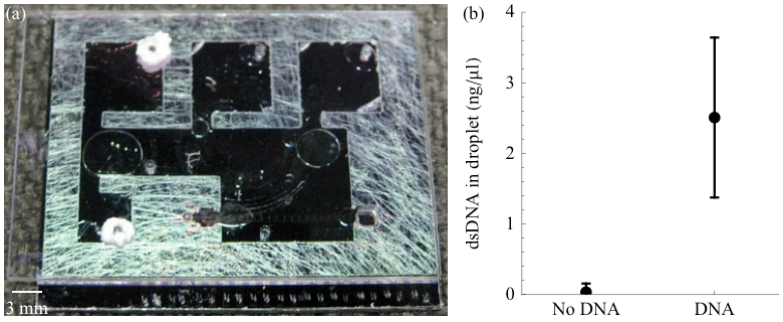

Figure 4. (a) Top-down view of the device, cf. Fig. 2a. After 60 °C elution, the GuSCN salt in lysis buffer and wash buffer 1 precipitated near the pipette ports. (b) dsDNA in the eluent droplets is determined by PicoGreen to be 2.5 ± 1.1 ng/µl (95% CI, n = 3.) In the control experiments, no DNA is added during the binding step.

CONCLUSION

Results from the initial development of a purification-to-quantification fluidic device are presented. In particular, a key feature of the device is demonstrated: the use of EWD droplet actuation to manipulate the eluent generated by an immiscible phase filtration DNA preprocessor. Compared with previous implementations, the feature will allow the concurrent quantification of more pathogen types. Also, relative to the concentration before binding, dsDNA in the eluent droplets is enriched by 13x. Thus, the detection limit is correspondingly improved. Furthermore, the purification power is currently estimated at > 40x, and it is feasible to achieve 10^3x per wash. Lastly, the ITO/PEDOT:PSS/acrylic top plate is developed to withstand > 100 °C. Later on, improved automation and sample-to-answer time would be brought by the inclusion of on-chip heaters and photodetectors.

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