INTEGRATING MULTIPLEXED PCR WITH CE FOR DETECTING MICROORGANISMS

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ABSTRACT. This paper describes an integrated plastic microfluidic device for detecting microorganisms. The device was designed to integrate heaters for polymerase chain reaction (PCR), microfluidic valves, and channels for sample injection and capillary electrophoresis (CE). The heaters, as well as the electrodes for CE, were screen-printed onto the device. *In-situ* gel polymerization was employed to form microfluidic valves that separate the PCR mixture from the CE separation medium. PCR was conducted in a channel reactor with a volume ranging from 30 to 500 nL, and four-plex PCR was successfully implemented in the device. After PCR, amplicons (PCR products) were electrokinetically injected through the gel valve, followed by CE. Two model microorganisms, *Escherichia coli (E. coli)* and *Salmonella*, were chosen to demonstrate the functionality of the device.

KEYWORDS: PCR, gel valve, plastic microfluidic device, bacterial detection

INTRODUCTION. Detection and identification of microorganisms are important for medical diagnostics, food/water safety testing, and biological warfare defense. Among several methods, nucleic acid-based genetic analysis, typically via PCR, has been increasingly used [1-2]. PCR products can be analyzed using either off-chip gel electrophoresis [3-4] or integrated CE analysis [5-7]. Incorporating PCR with CE in a single plastic device is attractive because of improved reliability and elimination of cross contamination due to the device being disposable. One of the challenges, however, is to integrate a valve that separates the PCR region from the separation medium to prevent mixing during thermal cycling [7].

In this report, we present an integrated plastic microfluidic device fabricated to detect microorganisms. We developed an approach to use a locally polymerized gel plug as a passive valve that separates the PCR region from CE analysis. We successfully implemented 4-plex PCR in the device and demonstrated the detection and identification of two model microorganisms, *E. coli* and *Salmonella*.

EXPERIMENTAL. The device was made from poly(cyclic olefin). The fabrication protocol has been previously described [8]. **Figure 1** shows the layout of the device. Referring to the expanded view of ID-6 at the bottom of Figure 1, Wells 5 and 6 are for introducing a sample into the PCR channel reactor between them. Well 7 is for the introduction of a DNA sizing ladder, which serves as a calibration standard to accurately identify the amplicons according to their sizes. Wells 8 and 4 are for loading a sample

plug into the separation channel, while Wells 1 and 3 are for running the separation. Well 2 was not used in this work. Integrated electrodes with contact pads function as CE driving electrodes by connecting to high voltages via "pogo" pins. The total volumes of the PCR channels in ID-1, 2, 3, 4, and 6 are 500, 570, 54, 29, and 84 nL, respectively.

To fabricate microfluidic gel valves, a photoinitiator, hydroxyl-cyclohexylphenylketone, was prepared in acrylamide/bis solution. The monomer solution was introduced into the entire device. After a photomask was placed on the device to define the exposed area where microfluidic valves were desired, the device was irradiated by a UV light. The monomer solution in the exposed areas polymerized to form a gel plug, whereas the monomer solution in unexposed regions does not polymerize, and it can be replaced with the desired buffers and reagents.

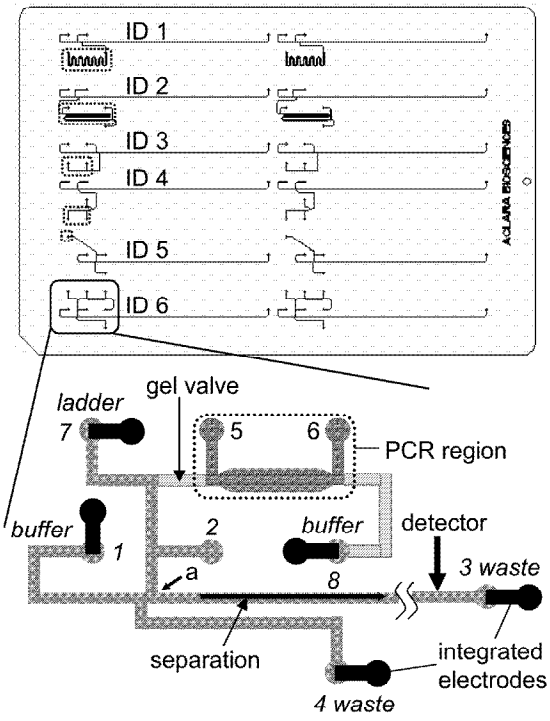

Figure 1. *Top: Layout of six microfluidic device designs on a single plastic device (12.8 x 8.5 cm); the heated region for PCR is indicated by dotted lines. Bottom: The most studied of the 6 designs, ID-6, is detailed in the expanded view, which shows gel valves in cross hatching, the PCR zone in diagonal shading, and printed ink electrodes in solid black.*

The setup for PCR thermal cycling consists of a heater, power supplies, a temperature sensor (RTD), a fan, and a computer. The heater and the cooling fan were controlled by a board from National Instruments. To fabricate heating resistors and CE driving electrodes, silver/graphite inks were screen-printed onto a poly(cyclic olefin) film. After being cured, the ink pattern on the film was aligned and thermally laminated onto the device.

A PCR reaction mixture was prepared containing roughly 4.5 x 10^3 copies of each of the target templates. The PCR mixture was filled into the entire PCR region. After filling the other channels and wells with appropriate reagents, all wells were sealed using PCR tape. PCR was carried out using temperature cycles including at 95 °C for 5 min., 35 cycles of (94 °C for 45 s, 56 °C for 30 s, and 72 °C for 45 s), and at 72 °C for 5 min.

RESULTS AND DISCUSSION

Plastic Devices. Each design in Figure 1 was devised to explore different concepts. ID-1 and -2 were designed for spatially resolved multiplexed PCR reactions. They have long serpentine channels in which PCR primers may be locally immobilized in different zones; this spatial separation of primer sets prevents primers from cross-talking with one other. ID-3 and -4 have a short PCR channel for a single PCR reaction mixture. ID-4 has an extra reservoir to allow the introduction of a DNA sizing ladder as discussed below. ID-5 accomplishes PCR in a well instead of a channel; this provides a good comparison with more traditional PCR conducted in tubes. All these designs were tested and found to be functional.

Integrated Heaters. The integrated, localized ink heater improves the rate of thermal cycling due to low thermal mass, thus speeding analysis. The low thermal conductivity of plastics was mitigated by using a film of ~100 μm thickness. Integration of screen-printed ink resistors potentially leads to low-cost mass-production, and provides intimate thermal contact. In addition, screen-printed ink contacts can also function as driving electrodes for CE.

We compared an ink heater with a commercial thermal cycler. Our results indicate the temperature ramp-up speed using the ink heater is roughly 4 times faster than a conventional commercial thermal cycler. The higher speed allows less time for mismatching that can occur between primers and targets; thus, fewer false positive results should be obtained.

Microfluidic Valves. We explored the concept of using an in-channel plug of gel as a pseudo closed valve during amplification and as an open valve during injection after PCR [9]. The performance of the gel valve was evaluated by measuring the degree of leakage. A device with a gel valve was subjected to thermal cycling with fluorescein added to the solution; the length of fluorescein that diffused through/into the gel was taken as an indication of the potential for leakage. In order to see the fluorescence image, a high concentration (10 mM) of fluorescein was used in this experiment. The actual leakage for amplicons should be significantly lower due to their larger size and much lower concentrations (0.1-100 μM). **Figure 2** shows two images taken before the 1st cycle and after the 30th cycle of the PCR reaction. The data show about 2 mm of fluorescein diffusion into the gel after 30

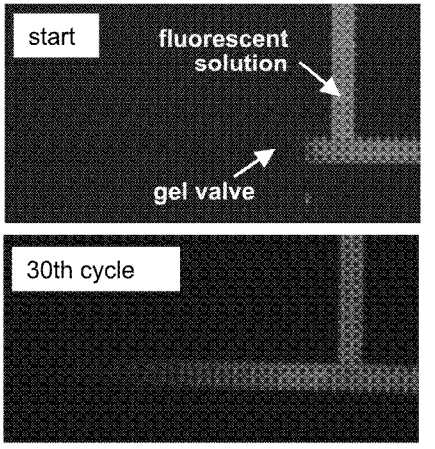

Figure 2. *Images of the interface between a gel valve and the PCR region filled with fluorescein, before the 1st cycle and after the 30th thermal cycle of PCR.*

cycles, which can be managed by designing CE components slightly more than 2 mm away.

Multiplexed PCR, CE and Bacterial Detection. Multiplexed PCR was conducted in the device by combining 4 sets of primers and targets. After PCR, CE separation of amplicons was carried out in a mixture of 1.5% HPC and 0.4% HEC in a Tris-Borate buffer at pH 8.4. An intercalating dye, thiazole orange (TO), is admixed to the separation medium. All double-stranded DNA complexes with TO, and thus can be detected by laser-induced fluorescence.

Two food-borne pathogens, *E. coli* O157 and *Salmonella typhimurium*, were chosen to demonstrate the integration of all microfluidic components and detection of microorganisms. The primer sets designed for them and the resultant amplicons of 232, 429, and 559 bp are specific for *E. coli*, all types of *salmonella*, and *typhimurium* only, respectively, allowing differentiation among them and from other microorganisms. **Figure 3** shows the electropherogram obtained from the device; all expected amplicons were produced. The primer set for genomic DNA K562, which results in an amplicon of 320 bp, functions as a positive control to avoid false negatives due to potential PCR failure. A fluorescent molecule, A411, was used as an internal standard to correct for any changes in migration time between runs.

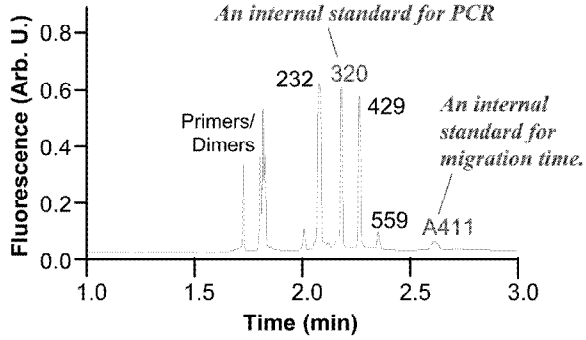

Figure 3. *Demonstration of the functionality of the integrated device and the interoperability of all microfluidic components. Electropherogram was obtained from 4-plex PCR.*

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