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Polydimethylsiloxane based Microfluidics Flow PCR Sensor for *Bacillus Anthracis* using a Disposable Electrode

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Abstract

Conventional Polymerase Chain Reaction (PCR) has been used widely in the clinical diagnostic and environmental monitoring where it still possesses larger volume and thermal mass leading to a slower PCR amplification for ultimate target detection. Microchamber stationary PCR is another alternative of conventional bench-top PCR in principle where the reaction chamber is cycled between two to three temperatures and a critical optimization of the system thermal mass is obvious to attain optimum reaction times and power consumption. Whereas our continuous flow through PCR system is independent of system thermal mass but depends mostly on the flow rates of PCR mixture in a microchannel for optimum target amplification along with the prevention of sample evaporation. In this report, a Polydimethylsiloxane (PDMS)-glass based two temperatures PCR microfluidic module was fabricated by soft-lithography and the *Bacillus Anthracis* target specific amplicon was detected using a disposable electrode instantly within 23 min with the detection limit of 10^6 copies/mL. Electrochemical sensing was done by Linear Sweep Voltammetry (LSV) and Hoechst 33258 [H33258, 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi(1H-benzimidazole)] - a DNA minor groove aggregating, redox active species - was used as electrochemical reporter. LSV was performed directly on microfluidics post-PCR products without further purification or immbolization within 60s of PCR completion. DNA-Hoechst 33258 aggregation due tominor groove binding led to a significant drop in the peak oxidation current intensity of redox molecule. We believe our sensor has the potential to integrate into the multiplexed, real-time microfluidics reusable PCR electrochemcial platform with further improvement in the future.

Keywords: Biosensor, Hoechst 33258, PCR

1. Introduction

Accurate detection of microbial pathogens is indispensable for Point Of Care (POC) diagnosis and also for food safety. So far, researchers have discovered numerous conventional ways for the detection of pathogenic microorganism but handling of chip based techniques has several benefits in terms of speed, cost, automation and accuracy[1-4]. We all know that for the detection of microorganism, the detection assays must be sensitive and specific which capable of detecting low concentrations of target species. Usually market oriented chemical

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detectors can detect chemical agents which might create risk to human, but biological detectors can only rarely detects pathogens directly from the samples which may cause a risk to human health. The main constraint to detect microorganism is its very low concentration and thus the target pathogen has to be cultured or concentrate to get their number up to a detection level[5-8].

Microfluidics is an active and productive area of research[9]. In the beginning silicon and glass based microfluidic devices were common instead of their time-consuming fabrication process[10,11] but the development of soft lithography and microfluidics large scale integration[12,13] has offered a variety of polymeric materials for microfluidic device construction which include Polydimethylsiloxane (PDMS),[12,14,15] Polymethyl Methacrylate (PMMA), Polycarbonate (PC), Polyethylene Terephthalate (PET), Polyurethane, Polyvinyl Chloride (PVC), and polyester[16].

Among these inexpensive PDMS has become the material of choice due to amenability to soft lithography, ease of patterning, optical transparency, flexibility and gas permeability. Though the innate hydrophobicity of PDMS surface impedes immediate use of PDMS microfluidics devices without surface processing, it still allows simple, easy to make or replicate planner microfluidic systems which are not easy to break.

Chip based microfluidics systems are amenable to integration with other DNA processing and analysis steps in micro total analysis system (μ-TAS)[17,18]. Usually in continuous flow PCR (CF-PCR) the reaction mixture passes through zones of alternating temperature corresponding to denaturation, annealing and extension. The CF-PCR avoids temperature cycling of the entire device and leads to faster heat transfer and higher throughput than batch PCR microfluidics chambers[19]. The heating and cooling rates for CF-PCR microfluidics are confined not by the system thermal mass but rather by the flow velocity of PCR mixture in a microchannel. Here the PCR sample solution does not suffer from large evaporation at high temperatures. In chamber stationary PCR microfluidics it lacks the flexibility to change the reaction rate resulting in more cycling and heating time. To reduce the reaction time and power consumption, the system thermal mass must be optimized considerably. Electrochemical approaches are preferred as it requires minimal instrumentation and it can be easily integrated with microelectronics into a chip-based format[20]. Hybridization-based detection schemes generally require single-stranded DNA (ssDNA) targets for which we use asymmetric PCR to generate ssDNA amplicons. Yeung et al. have shown multiplexed detection of asymmetric PCR through electrode specific probe immobilization[21]. Regrettably, asymmetric PCR usually provides linear rather than exponential amplification, requires probe immobilization, significantly longer reaction time and it is less efficient than symmetric PCR[22,23]. Label based electrochemical analysis using H33258 required immobilization of "probe" on the electrode sensing layer. In contrast, our method can avoid probe immobilization as H33258 interact with DNA to produce less H33258 molecule onto the electrode surface. So the anodic current peak of DNA-H33258 complex decreases in proportion to dsDNA titration. In this paper we have described a microfluidics device for the continuous flow PCR using PDMS and glass (Figure 1). The model organism *Bacillus Anthracis* have been chosen for the amplification of target DNA and off chip redox molecule based electrochemical detection without any immobilization. Here for the first time the microfluidics PCR amplicons were analyzed in solution based on the DNA-Hoechst 33258 interaction directly on to the disposable

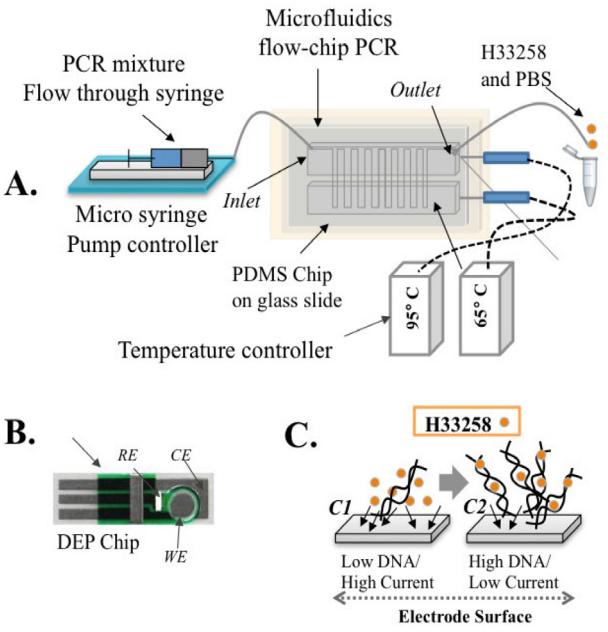

Figure 1. A: The experimental setup for the flow-injection of PCR solution; and the channels were heated with two temperatures heat-block (65° C and 95° C). The amplicons were collected and detected off chip using disposable electrodes. B: Disposable carbon disposable-electrode. C: Principle of electrochemical detection based on PCR amplicon-H33258 interaction of disposable electrode.

electrochemical printed chip without any purification step using the Linear Sweep Voltammetry (LSV).

2. Experimental

2.1 Microchip Design and Fabrication

We have used standard soft lithography process to fabricate PDMS (Dow Corning, Midland, Michigan) microfluidic PCR chips. The microfluidic channel was 300 µm wide and 200 µm deep (Figure 2) 24 with 500-µm diameter inlet and outlet holes to inject PCR reaction mix and to collect post-PCR products. Fluorinated Ethylene Propylene (FEP) tube (0.15±0.05-mm i.d.) (BAS Inc., Tokyo, Japan) was used to connect in lets and outlet. Small amounts of PDMS were used at base of tubes to prevent liquid leaks. Irreversible bonds between glass slides and PDMS chips were created by oxygen plasma. We also have used microsyringe pump (KD Scientific Inc., Holliston, MA, USA) and a 1-mL-volume syringe (Terumo Co., Tokyo, Japan) to inject the PCR mixture. Aluminum block, 10×10×70 mm in dimension, was connected to a cartridge heater and a temperature sensor (Kyushu-Nissho Co, Fukuoka, Japan) were used for PCR's two temperature cycling. The temperature of the blocks was controlled within ±1°C by a thermocouple of type Φ 1.6 K and a temperature controller (Kyushu-Nissho Co, Fukuoka, Japan). The mineral oil (Sigma, USA) with a density of 0.84 g/mL at 25°C was used in this experiment as vapour barrier. Bovine Serum Albumin (BSA) of 1% has been used as a coating agent to reduce non-specific adsorption of PCR cocktail to the glass and PDMS surface.

2.2 Flow Chip PCR Primers and Anthrax DNA Preparation

For our experiment we have chosen the primer sequences of pX02 from the plasmid of Anthrax (*Bacillus anthraces*). Plasmid pX02 encodes a group of genes involved in capsule synthesis (capA, capB and capC). Our primer sequence is specific for CAP (Genebank accession No. M24150) specific gene. The primers cross-reactivity have been carefully tested and verified before chip experiments. To achieve target DNA, the *B. Anthraces* was cultured and grown on Brain heart infusion (Difco, Japan) medium overnight at 37°C. The grown anthraxes were heat-treated and then directly used for flow chip PCR. The primers used in the *B. anthracis* amplification were 5'-GACGGATTATGGTGCTAAG-3' (for) and 5'-CAATAGCTCCTGCTACAAAT-3' (rev) for the amplification of a 179-bp fragment of the CAP gene.

2.3 PCR Protocol for Chemical Amplification

For the optimization of shuttle PCR, initial denaturization of 95°C, and annealing/extension of 60°C to 65°C were tested. Using the bench-top PCR system of 30 to 50 cycles, different copy numbers/reaction was analyzed electrochemically (data not shown). Finally the temperature was

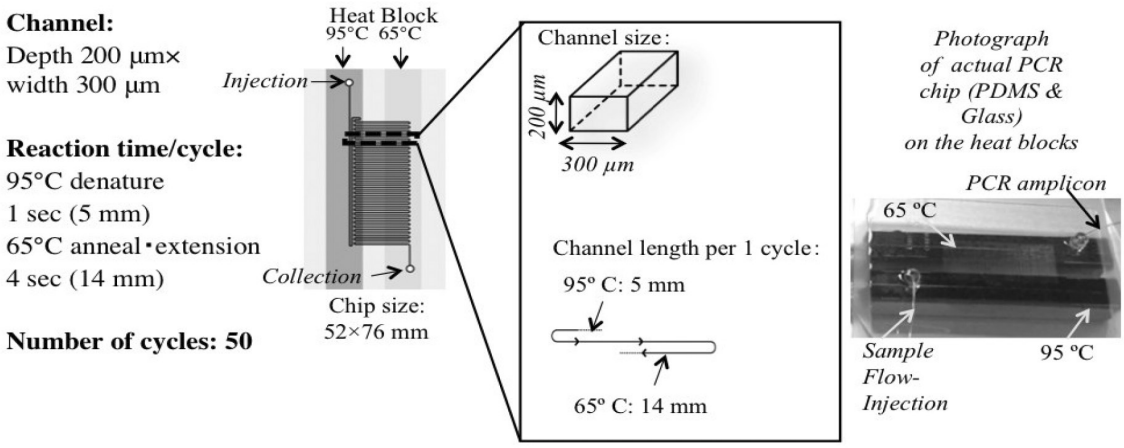

Figure 2. Layout of the flow-through PCR chip on two different temperatures of aluminum blocks (left). Photograph of PDMS-glass chip with its inlet and outlet (right).

chosen as 95°C for denaturation and 65°C for annealing/extension. Beside the template DNA, PCR mixture for continuous flow PCR contained 1.25 µL of SpeedSTAR™ HS DNA Polymerase (Takara Bio Inc., Japan) in 2.5 µL volume 10× Fast Buffer I (Takara Bio Inc., Japan), 0.2 µM of both the forward and reverse primers, 2.5 mM of each dNTP and, 1% (w/v) BSA to block unspecific adsorption of reagents to PDMS surface. We have started with injection of mineral oil into the microchannels followed by the flow of 1% BSA through the channel. At this stage the heat block was concurrently switched on with full temperature set for flow chip PCR. Microfluidic channels were so designed to make sure the flow of PCR mixture through two different temperature zones alternatively for 50 cycles. Finally, we injected the PCR assay mixture. Flow rate for PCR mixture varied between 11 to 17µL/min and the optimum rate of 15µL/min was used for further experiment. The PCR amplicons were collected in a 0.5 mL polypropylene tube and used for electrochemical measurement within 60s.

2.4 Electrochemical Measurement using Disposable Electrodes

All electrochemical studies were performed in conjunction with Linear Sweep Voltammetry (LSV) using an Autolab PGSTAT 12 electrochemical analysis system (Eco Chemie, The Netherlands) in connection with its General Purpose Electrochemical System (GPES) software. All experiments were carried out at the ambient temperature of the laboratory (23–27°C). Measurements were the average of at least three repeated measurements. LSV was performed while scanning from 0.0 V to 1.0 V with a step potential of 0.00244 V and a scan rate of 100 mV/s. The PCR products were diluted to 2× with PBS (pH 6.0), and then they were mixed with 20 mM of H33258. The changes in the anodic peak current responses were recorded and processed using the software Autolab PGSTAT 12. For our study LSV has been chosen as it showed fast and reproducible anodic oxidation peaks during measurement.

3. Results and Discussion

The injection of PCR cocktail solution into the tiny sized hydrophobic channel is difficult due to the high surface-to-volume ratio. As we know to attach the glass irreversibly with the PDMS chip oxygen plasma treatment is necessary to provide –OH group onto the PDMS surface. This plasma treatment therefore reduces the hydrophobicity of the microchannel surface and makes it more suitable for continuous flow microfluidics PCR. However, this hydrophilic surface state is hard to maintain for longer time and therefore we worked with our chips within a week of oxygen plasma treatment[25]. On the other side, to reduce the adsorption of the PCR components onto the surface of PDMS, we added BSA as a blocking agent for each flow chip PCR. It was not unexpected to observe small air bubbles at the high temperature zones at the initial phase of the reaction but those air bubbles were disappeared immediately. We believe that due to the permeability of the PDMS to air those air bubbles were disappeared and did not interfere the flow of the PCR solution.

In our previous study, we developed an electrochemical biosenor that did not rely on the tedious probe immobilization steps onto the electrode surface. We observed that the anodic current decreases in proportion to the titration of the dsDNA with H33258[26-28]. The H33258 and DNA interaction caused condensation as confirmed by Saito et al. using atomic force microscopy[29]. Besides DNA-H33258 minor groove binding phenomenon also observed by Vega et al.[30]. For our study, we have verified that there were no other-electroactive compounds in the PCR mixture that may yield anodic overlapping signals at the peak potential of H33258 (data not shown). In our study nuclease-free water was used as a negative controls. Besides, any non-specific and unamplified microbial DNA is negligible compared to the PCR amplified product by the conventional fluorescent and gel observation. We have not seen significant peak current changes in comparison with the case of using water as a template and therefore water was used as the negative controls. We have also checked the cross reactivity of our primers with other Bacterial species (*Bacillus Subtilis*) both on bench-top and the on chip analysis and have not seen any amplification as there were no target sequence like CAP gene of *B. Anthracis* in *B. Subtilis*.

Flow-rate of PCR mixture through the channel of a microfluidic-flow-PCR chip is the most critical factor for optimum thermal cycling to ensure successful amplification and to determine the duration necessary for PCR amplification (Figure 3). At higher flow rate, the inefficiency in thermal cycling and the formation of bubbles in the flowing PCR mix becomes insurmountable issues while at lower flow-rate, the successful amplification takes undesirably longer time. Therefore, the flow-rate optimization is necessary to ensure adequate amplicon for the

terminal electrochemical detection of the PCR product. In this study, through our microfluidic-flow-PCR chip, four different flow-rates of PCR mixtures (Figure 3), *viz.* 11, 13, 15 and 17 µl min^{-1}, have been investigated. The flow-rate of 17 µl min^{-1} produced undistinguishable number of target copies for electrochemical detection relative to the negative control i.e. samples without PCR amplification. However, by bringing down the flow-rate marginally, enough amplicons could be obtained from PCR that generated a clear drop in the oxidation peak current. Subsequent enhancement in PCR was also observed with additional reduction in flow-rate. Keeping the issues of bubble generation at high flow-rate and the longer duration for PCR at low flow-rate in mind, we took 15 µl min^{-1} as the optimum rate for the flow-chip PCR and conducted all subsequent amplification at this flow-rate.

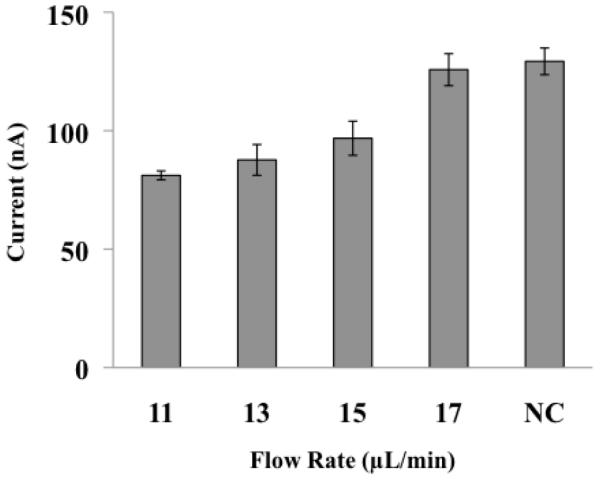

Figure 3. Flow-rate optimization for microfluidic flow chip PCR using 10^8 copies/ µl. Compared to negative control, the flow rate 15 µl min^{-1} stands out to be the optimum for producing enough PCR amplicons for quickest possible detection.

We determined the minimum flow through time for needed for our chip to create enough amplicons of our target at the optimum flow rate of 15 µl min^{-1}. This in turn defined the shortest time needed for the detection of target gene using this microfluidic-flow-PCR chip. The gel electrophoresis of the amplified samples collected after different flow-through times using the chip showed the presence of PCR amplicons for as low as 19 to 21 minutes for 10^8 copies per µl. But for confirmed electrochemical detections, we took amplicons independently at 23, 25 and 27 minutes of flow-through times for which gel image shows very good amplification. The drop in the peak oxidation current for amplicons at 23 minutes of flow through time was clearly detectable (Figure 4a) compared to negative control but at 25 minutes it was much clear while longer flow-through time (27 minutes) did not bring about significant changes in the anodic peak intensity (Figure 4b and 4c). Under the optimized condition, the microfluidic-flowchip PCR could produce detectable amplicon from a template concentration of 10^6 copies per µl of flow-through PCR mix (Figure 5). At higher template concentrations, due to the availability of higher number of templates, the time of detection could be reduced but when the template concentration fell below 10^6 copies per µl, no detectable amplicon was observed even at longer flow-through time. In Figure 5 (right), given is the electrochemical detection of PCR amplicons from the chip

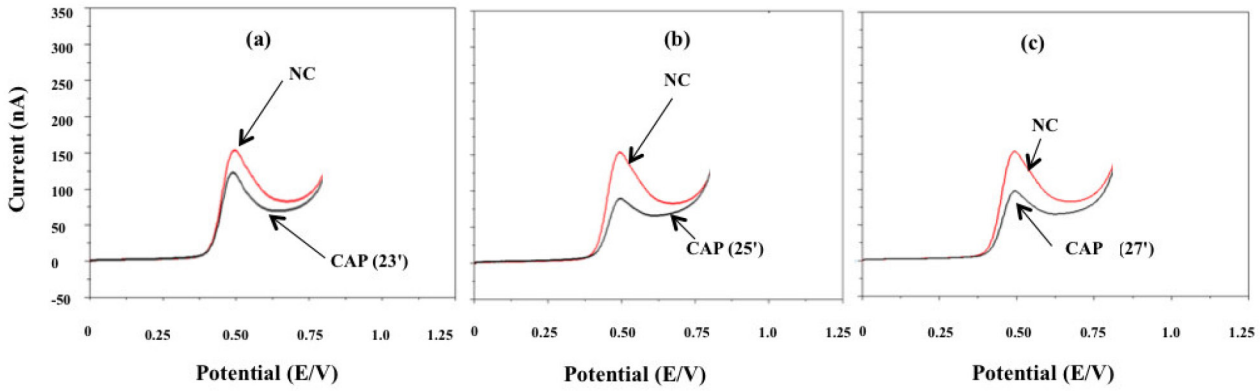

Figure 4. The detection time of anthrax DNA (10^8 copies/µL) based on the minimum flow-through time that produce enough amplicons for a clear drop in the peak oxidation current during electrochemical detection. NC, Negative control as a nuclease free water. Whereas CAP, amplicons obtained at different time interval and detected electrochemically using LSV as detection mode transducer using disposable electrodes.

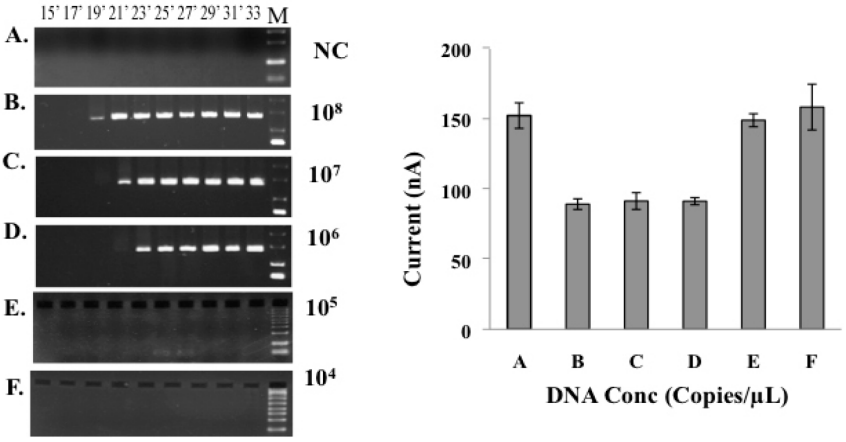

Figure 5. The limit of detection based on the template concentration in the flow-through PCR mix for the chip. The gel electrophoresis data of amplification using different template concentrations (Left), The electrochemical detection of amplicons in 23 min. for different template concentrations at optimum flow-rate and flow through times indicating the limit of detection for the amplicons from this chip is 10^6 copies of target DNA per µl of PCR mixture. The study shows higher template conc. (10^8) amplify faster (left, B in 19 min.) and lower template conc.(10^6) amplify comparatively slower (left, D in 23 min.).

at optimum detection time of 23 minutes, which clearly showed that at the target concentration equal to or above 106 copies per µl, this chip platform was effective in successful amplification, which gives the limit of detection for this chip platform. Our results also suggest that BSA alone could not solve the problem associated with the microfluidics inner channel's adsorption of polymerase, primers, and Anthrax DNA. Alternatively, we think, surface treatment such as poly (vinylpyrrrolidine) (PVP) coating may increase the sensitivity and dynamicity of this platform[24,31].Besides, this continuous flow PCR chip's future integration like sample preparation, DNA amplification and electrochemical detection can be incorporated on a single system and that can perform the analysis in a continuous-flow manner.

4. Conclusion

The results reported herein represent the first example of performing flow-chip PCR for *B. Anthracis* specific gene amplification and disposable electrode based electrochemical detection. Our detection limit was 10^6 copies/uL of *B. Anthracis* which was detected electrochemically within 23 min. Our electrochemical detection method avoided the surface functionalization process which is often the bottleneck in the development of true handheld electrochemical biosensors. As the threat from widely scattered biological warfare agents usually requires new smart instrumentation systems for autonomous monitoring so it is not too far for the future that hundreds of this type flow-chip PCR could run in parallel for continuous and high throughput analysis. We are also optimistic that there is a potential to integrate the flow-chip PCR into multiplexed, real–time reusable analysis platform on which our group is now focusing on[32–34].

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