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A circular ferrofluid driven microchip for rapid polymerase chain reaction

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In the past few years, much attention has been paid to the development of miniaturized polymerase chain reaction (PCR) devices. After continuous flow (CF) PCR chip was introduced, several CFPCR systems employing various pumping mechanisms were reported. However, the use of pumps increases cost and imposes high requirement on microchip bonding integrity due to the application of high pressure. Other significant limitations of CFPCR devices include the large footprint of microchip and the fixed cycle number which is dictated by the channel layout. In this paper, we present a novel circular close-loop ferrofluid driven microchip for rapid PCR. A small ferrofluid plug, containing sub-domain magnetic particles in a liquid carrier, is driven by an external magnet along the circular microchannel, which in turn propels the PCR mixture through three temperature zones. Amplification of 500 bp lambda DNA fragment has been demonstrated on the polymethyl methacrylate (PMMA) PCR microchip fabricated by CO₂ laser ablation and bonded by a low pressure, high temperature technique. Successful PCR was achieved in less than 4 mins. Effects of cycle number and cycle time on PCR products were investigated. Using magnet as the actuator eliminates the need for expensive pumps and provides advantages of low cost, small power consumption, low requirement on bonding strength and flexible number of PCR cycles. Furthermore, the microchip has much simpler design and smaller footprint compared to the rectangular serpentine CFPCR devices. To demonstrate its application in forensics, 16-loci short tandem repeat (STR) sample was successfully amplified using the PCR microchip.

Keywords: Ferrofluid; PCR; microchip; PMMA; circular; forensic

Introduction

The Polymerase chain reaction (PCR) process is widely used as a molecular biological tool to replicate DNA, and can create copies of specific fragments of DNA by cycling through three temperature steps. Each temperature cycle can double the amount of DNA, and 20–35 cycles can produce millions of DNA copies. Recently, much attention has been paid to the development of miniaturized PCR devices^{1–3}. All kinds of PCR microfluidic technologies have facilitated DNA amplification with much faster rates as a result of smaller thermal capacity and larger heat transfer rate between the PCR sample and temperature-controlled components⁴. Two basic types of PCR devices have been described, namely temporal PCR devices and spatial PCR devices⁵. In a temporal PCR device, the sample solution is introduced into a small chamber. Thermal cycling is realized by heating and cooling the reaction chamber repeatedly according to required temperatures of the denaturing, annealing and extension processes. Such small chambers are difficult and expensive to fabricate, the temperature control system is usually bulky and the heating and cooling rates are relatively low⁶.

For spatial PCR devices, the PCR reaction mixture, instead of being held in a thermal cycling chamber, is pumped into a serpentine microfluidic channel and passes repeatedly through three spatially fixed temperature zones. In this concept, temperature at a specific location only needs to be controlled at a constant value. The first spatial PCR device was developed by Kopp *et al*⁷ and called continuous-flow (CF) PCR device. Since

then, a number of CFPCR systems based on both glass and polymer have been introduced. Currently, most researchers use syringe pump^{8–10}, peristaltic pump¹¹ or rotary pump¹² to control the flow of PCR solution through microchannels. Although successful DNA amplification has been reported for many CFPCR devices, some problems may ultimately hinder their widespread use. Significant limitations of CFPCR devices include the large footprint of microchip due to the long serpentine microchannel required for multiple PCR cycles and the fixed cycle number which is dictated by the channel layout. Moreover, the use of various pumps imposes high requirement on microchip bonding integrity. Liquid leakage has frequently been observed due to the large pressure involved. In addition, the high cost and bulky size of pumps make it difficult to realize true micro total analysis systems (μTAS).

One solution for the long microchannel of CFPCR devices is containing the flow in a closed loop. Krishnan *et al.*¹³ used temperature gradients in a 35-μL cylindrical cavity for both driving and thermal cycling of the PCR mixture. The mixture circulates vertically between 97°C and 61°C due to natural convection. This concept was further extended to close-loop designs with rectangular shape¹⁴, triangular shape¹⁵ and semicircular shape¹⁶. The major drawback of this concept is that the driven force based on natural convection is a body force, which is proportional to the volume and does not scale favourably in microscale. Consequently, the concept only works with devices in mesoscale with channel size on the order of millimeters.

Magnetic force can be used to drive the PCR mixture in a close-loop channel. Although magnetic force is also a body force, this actuation concept can be miniaturized due to the possible high magnetic field gradient in microscale. Hatch *et al.*¹⁷ previously reported a micropump using a ferrofluid plug as a piston, which is driven by an external magnet. Similar magnetic actuation was also reported later by Yamahata *et al.*¹⁸ Both of the devices were used for continuous pumping of liquids.

In this paper, we present a close-loop circular ferrofluid driven microchip for rapid PCR. An external magnet is used to drive a small ferrofluidic plug inside the circular microchannel, which in

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turn pushes the PCR reaction mixture to move around the circular microchannel and to travel through the three temperature zones. The number of thermal cycles can be varied by controlling the number of rotation cycles of the magnet. Cycle time of the PCR mixture is adjusted by changing the rotation speed of the magnet. Effects of cycle number and cycle time on PCR products were investigated. The microchip has much simpler design and smaller footprint compared to the rectangular serpentine CFPCR devices, and using magnet as the driving force provides the advantages of low cost, small power consumption, low requirement on microchip bonding integrity and flexible number of PCR cycles. Forensic application was demonstrated by performing amplification of 16-loci short tandem repeat (STR) sample on the PCR microchip.

Concept of ferrofluid driven method

A ferrofluid is a stable colloidal suspension of sub-domain magnetic particles in a liquid carrier. Each ferro-particle has an average size of about 100 Å (10 nm). These ferro-particles are specially coated with surfactant, a stabilizing dispersing agent to prevent agglomeration due to strong magnetic field gradient applied to the ferrofluid, as well as to maintain the colloidal and thermal stabilities, which are crucial to PCR applications. The ferrofluid used in our experiment contains 5% magnetic solid, 10% surfactant and 85% carrier by volume. The carrier liquid is synthetic ester oil, which is immiscible with the aqueous PCR reaction mixture. The viscosity, the density, and the surface tension of the ferrofluid at 25 °C are $\eta_{ff} = 0.406$ kg/ms, $\rho_{ff} = 1330$ kg/s, and $\sigma_{ff} = 32 \times 10^{-3}$ N/m, respectively. The actuation of droplets of this ferrofluid has been recently studied by Nguyen *et al.*¹⁹ In the absence of magnetic field, the ferro-particles are randomly distributed, thus the ferrofluid has no net magnetization. Conversely when magnetic field is applied to the ferrofluid, the ferro-particles will automatically orient themselves in neat alignment along the magnetic field and cause concomitant repulsion between adjacent particles. The magnetization of the ferrofluid responds quickly to any changes in the applied magnetic field and the moments randomize rapidly when the applied field is removed. In a gradient field, the whole fluid responds as a homogeneous magnetic liquid which move to the region of the highest flux. This means that ferrofluid can be precisely positioned and controlled by an external magnetic field. The forces holding the magnetic fluid in place are proportional to the gradient of the external field and the magnetisation value of the fluid. All these features allow the use of a ferrofluid plug to drive the PCR reaction mixture in a close-loop microchannel.

Experimental

System setup

Figure 1 shows a schematic diagram of the system set-up which includes temperature control system, a ferrofluid driven PCR microchip and an external permanent magnet. The temperature control system (Tri-X Pte Ltd, Singapore) consists of a proportional-integral-derivative (PID) temperature controller, sensors and three circular-arranged copper heating blocks. Each copper block is 55 mm high and 10 mm thick. Temperature can be set from 25 °C to 150 °C.

The copper blocks are separated by small air gaps to ensure the formation of three distinct and steady temperature zones. Thermocouple sensors were attached to each copper block for temperature monitoring. Unlike the parallel arrangement of the temperature zones in most flow-through microfluidic chips, the circular arrangement of the three zones allows the real realisation of the sequence of denaturation, annealing and extension instead of denaturation, extension and annealing. This prevents the denatured single-stranded DNA to re-hybridise in the extension zone, resulting in increased amplification efficiency.

A cylindrical neodymium magnet (15 mm high and 10 mm in diameter) coupled with a home-made stepper motor was mounted on top of the circular microchannel. The stepper motor was used to provide the rotating motion for the magnet, which in turn moved ferrofluid and PCR reaction mixture. A full PCR cycle was completed when the magnet turned one round. The speed of the magnet can vary from 1.3 to 7.5 rpm, corresponding to a linear velocity from 1.4 to 7 mm/s for PCR reaction mixture and a cycle time from 46 sec to 8 sec. The number of PCR cycles can be chosen arbitrarily by programming the rotation cycles of the magnet.

Microfabrication

The design of the microchip was much simpler compared to the rectangular serpentine CFPCR devices. It consists of a deep close-loop circular channel for PCR reaction and two shallow straight channels tangent to the circle for sample injection and collection. The PCR chip was fabricated in polymethyl methacrylate (PMMA) substrate by laser ablation.²⁰ The microfluidic pattern was designed using CorelDraw (Corel Co., Canada). The pattern was then sent to a commercial CO₂ laser scribe (Universal M-300 Laser Platform, Universal Laser Systems Inc., Arizona, USA) for direct micromachining on PMMA substrate. Inspection of scanning electron micrographs (SEM) indicated that the channel had a Gaussian-shaped cross section due to the Gaussian distribution of the laser beam energy. Access holes were also drilled by the same CO₂ laser to allow fluid access to the microchannels. To form the microfluidic device, the engraved substrate was then bonded together with a cover plate by a low pressure, high temperature thermal bonding technique under pressure of 20 kPa at 165 °C for 30 minutes.

As shown in Fig. 2, the outer dimension of the chip was 30 mm × 30 mm. The circular channel was 62.8 mm long (with a diameter of 20 mm), 200 µm wide and 250 µm deep, and each temperature zone was 21 mm long. The total volume was 2 µl, about one to two orders smaller than that of devices based on natural convection reported before. Two shallow straight channels were 10 mm long, 100 µm wide and 50 µm deep to reduce sample waste. Rectangular holes labelled as “thermal gaps”, were machined in a radial fashion to ensure proper heat isolation between different temperature zones. Small circular holes were drilled 200 µm away from the microchannel as sensing ports for measuring the temperatures around the loop. Access to the microchannel was provided by Teflon tubing attached to two holes drilled into the cover substrate.

Preparation of PCR mixture

To test the fidelity of the PCR microchip, a 500 bp fragment of bacteriophage lambda DNA was amplified. Forward primer is 23-mer (5'-GAT GAG TTC GTG TCC GTA CAA CT-3') with a melting point of 64.1 °C. The primer coordinates are 7131-7153 on the lambda DNA template. Reverse primer is 23-mer (5'-GGT TAT CGA AAT CAG CCA CAG CG-3') with a melting point of 70.3 °C. The primer coordinates are 7608-7630 on the template. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 2 × BSA (0.5 µg/µL), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP, 1 µM of each primer, 1 ng/µL lambda DNA as the PCR template, and 0.025 U/µL *Taq* DNA polymerase. All samples and reagents for PCR reaction were purchased from Research Biolabs (Singapore). The ferrofluid (APG S10n) used in the experiment was purchased from Ferrotec (CA, USA).

One important application of the ferrofluid driven PCR microchip is to amplify forensic DNA. DNA fingerprinting requires the co-amplification of sixteen loci, fifteen STR loci and Amelogenin. STRs are short repeated sequences appeared in human genes at different chromosomes. The ferrofluid driven PCR microchip were tested using Applied Biosystems Identifiler® STR analysis kit (Applied Biosystems, Foster City, CA, US) with 0.04 ng/µl 9947 female DNA template (Promega, Madison, WI, USA). Amplicon allele size ranges between 100 to 360 base pairs. Four fluorescent dyes (6-FAM, VIC, NED, and PET dyes) were used to label DNA fragments. All STR loci included in the Applied Biosystems Identifiler® STR analysis kit are co-amplified in a single PCR.

Procedure

As shown in Fig. 3, the temperature of the denaturation zone was set at 95 °C, annealing zone at 60 °C and extension zone at 72 °C. Temperatures inside the microchannel were measured using a K-type thermocouple (Tri-X Pte Ltd, Singapore).

The microchip was placed on top of the heating blocks. For amplification, following a 2-min flush with deionized (DI) water, the PCR mixture was injected using a glass syringe (Hamilton, NV, USA) through Teflon tube (Cole Parmer, Illinois, USA) fitted to the chip inlet hole. After the channel was completely filled, a small portion (0.5 µl) of ferrofluid was injected through the same inlet port. The inlet and outlet ports were then sealed with epoxy gel (3M, Minnesota, USA).

Upon introduction of magnetic field, the ferrofluid plug immediately moved with the rotating magnet. PCR reaction mixture was pushed around the circular channel and flowed through the three temperature zones continuously. The cycle time of PCR reaction mixture was changed by adjusting the rotational speed of the magnet and the number of PCR cycles was varied by selecting the number of rotation cycles of the magnet. After amplification, the epoxy gel was removed and the PCR products were propelled by a syringe and collected into a polypropylene tube at the outlet. Subsequent to each run, the microchip was washed by DI water and negative control of PCR was done to make sure that there was no carryover. The simplicity of the system should make it possible to use the chip as a disposable, thus the washing step is not necessary.

For amplified lambda DNA, the collected samples were analyzed using a 1.2% crosslinked agarose gel (Sigma Chemical Co, MO, USA) stained with Ethidium Bromide (EtBr) (Sigma Chemical Co, MO, USA). 0.5 µg of 100 bp DNA ladder (500 µg/ml) was run together with PCR products. The ladder can be used for approximating the mass of DNA and the estimated mass of 500 bp DNA fragment in 0.5 µg loading is 97 ng. UV emission from the product was imaged by Genesnap software (Syngene Pte Ltd, UK) and integrated intensities of the product bands were calculated using image integration software Genetools (Syngene Pte Ltd, UK).

The amplified 9947 STR loci were analyzed simultaneously in a single injection with ABI 3100 PRISM DNA analyzer (Applied Biosystems, Foster City, CA, USA). The DNA analyzer is a multi-color fluorescence-based DNA analysis system based on the proven technology of capillary electrophoresis. It is fully automated from sample loading to data analysis.

Results and discussions

Temperature distribution

As temperature control is one of the most critical factors in PCR, temperature distribution of the PCR microchip was studied. Since the dimension of the microchannel was very small, it was assumed that the temperature measured at the small hole 200 µm away from the channel wall would be a good estimate of the temperature within the microchannel. Fig. 4 (a) shows the points along the microchannel where temperature was measured. Points 1-6 were located in zone I (denaturation), points 7-11 (annealing) in zone II and points 12-15 in zone III (extension). Fig. 4 (b) shows the temperature measured along the circular microchannel and it is clear that three distinct temperature zones were created. Within each zone, the respective temperature was almost constant, changing sharply between the two adjacent zones. This could be attributed to the proper thermal isolation by air gaps and the small thermal conductivity of PMMA (0.17W m⁻¹ K⁻¹). As the cycling temperatures were precisely controlled, desirable temperature kinetics for PCR were acquired and PCR products with high throughput and high specificity could be obtained. Other DNA templates with different temperature requirement can also be amplified by changing the temperature setting.

Effect of cycle number

To demonstrate that PCR can be achieved by the magnetically driven concept, amplification of 500 bp lambda DNA fragment was conducted on the PCR microchip. The effect of cycle number was studied. This was achieved by varying the number of rotation of the magnet. The rotation speed of the magnet was selected to be 2.2 rpm, giving a cycle time of 27 s, i.e. 9 s at each temperature zone. It would take two hours to finish a 30-cycle PCR on a conventional thermal cycler, while it only took 13.5 min to finish the amplification on microchip

as the result of smaller thermal capacity and larger heat transfer rate between the PCR sample and heating blocks.

Figure 5 (a) shows the effect of cycle number on the efficiency of PCR. Using the image integration software, the integrated intensities from the PCR product gel bands were normalized with respect to the integrated intensity obtained from the 500 bp fragment and the curve is shown in Fig. 5 (b). The PCR product from the microchip started the exponential increase at the cycle number of 8 and showed a trend to reach saturation gradually. The curve began to level off around the circle number of 20, indicating that efficiency of PCR was reduced at large cycle number due to the fast decrease of the concentration of PCR mixtures. As demonstrated by Hu *et al.*²¹, for larger concentration of DNA template, PCR product became detectable at smaller cycle number and also need less cycles to reach saturation. Since the thermal cycle number is not fixed in the microchip, optimum cycle number can be chosen for different DNA concentrations.

Effect of cycle time

The velocity of the PCR mixture through the microchannel determines the residence time in each temperature zone and the total cycling time of PCR. The cycle time is limited by the synthesis rate of the DNA polymerase. Above an upper limit, the PCR process will not be fully performed and a decrease in the amount of PCR products will be observed. Since the extension rate of *Taq* polymerase is 60-100 nucleotides/s at 72 °C in conventional PCR²², an extension time of 9 s is considered sufficient for a 500 bp PCR product. The influence of the extension time on the amplification efficiency was investigated in order to minimize the time for the whole PCR process. Microchip PCR was performed with 25 cycles at different cycle times. The conditions are summarized in Table 1.

Fig. 6 (a) shows the effect of cycle time on the efficiency of PCR. The normalized intensities of the PCR product are shown in Fig. 6 (b). The amount of the PCR products from the microchip increased exponentially first and then reached plateau with the increase of the extension time. The curve started to saturate at extension time of 9 s, which verifies that the optimum extension time of amplifying the 500 bp DNA fragment is around 9 s. Therefore, depending on the size of fragment that is amplified, proper cycle time can be selected by controlling the rotation speed of the magnet to achieve fast amplification. Limited by the velocity of stepper motor, the shortest extension time attainable by the system is 2.6 s, and at this speed, DNA fragment less than 200 bp could be completely amplified. For the PCR microchip, the extension reaction is strictly restricted to the extension region since there is no final extension step. We believe that the amplification efficiency for larger DNA fragment can be further increased by adding a pre-denaturation reactor at the inlet and a post-extension reactor at the outlet.

Amplification of 16-loci STR sample

A volume of 1.5 µl PCR reaction mixture and 0.5 µl ferrofluid were injected through inlet. 25 cycles of amplification was

completed in 13 mins with flow velocity of 2 mm/s and cycle rate of 31.4 sec/cycle. The time was greatly reduced compared to almost 2 hours when using conventional thermal cycler. Triplicate amplification was carried out and negative controls were conducted in between template-containing runs.

Electropherograms for 9947 female DNA and negative control are shown in the Electronic Supplementary Information. For 9947, all 16 allele were successfully amplified. Although relative fluorescence units (RFUs) were mostly under 100, which were relatively lower than those achieved from conventional thermal cyclers, peak heights were very balanced and no obvious imbalanced peak was observed. The low RFUs can be overcome by increasing amplification cycles. RFUs of negative control were all under 10, proving that there was no carryover and cross contamination.

Conclusions

In this paper, a novel circular ferrofluid driven PCR microchip has been developed and successfully demonstrated by performing PCR amplification of a 500 bp lambda DNA fragment and a 16-loci forensic DNA sample. The intensity of PCR product increases exponentially with the number of cycles until a saturation point. As the thermal cycle number in the PCR microchip was adjustable by controlling the magnet rotation cycles, an optimum cycle number could be chosen for different DNA concentrations. The cycle time of the PCR mixture was controlled by the rotation speed of the external magnet. The microchip has much simpler design and smaller footprint compared to the rectangular serpentine CFCR devices, and this simple method to carry out PCR thermal cycling using ferrofluids in a close-loop microchannel potentially offers a big step toward a real micro TAS for its small power consumption, ease of operation and low cost. It is expected that the presented device concept could be widely used in forensic, clinical and biotechnological applications. The problem of low sample throughput can be tackled by designing a series of concentric circles on one microchip. As the dimension of microchannel is small compared to the magnet, one magnet is adequate to drive PCR reaction mixture in all the microchannels. The freedom of choosing different times for the three temperature zones could be realised by changing the geometry of microchip layout. Other than annular loop, triangular shape is another option. By designing different lengths at three temperature zone, different cycling time is achievable. The pre-denaturation and post-extension on chip is also possible by adding two reactors at the inlet and outlet. We have recently developed a rotate-and-slide mechanism that allows the external magnet to move along an arbitrary closed path, which can replace the current simple circular path. These are the future directions of our research. In the future, we will devote to the development of parallel ferrofluid driven PCR microchip and the integration of PCR microchip with capillary electrophoresis (CE) microchip.

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Fig. 1 Schematic drawing of the PCR microchip with heating blocks and magnetic source.

Fig. 3 Concept of ferrofluid driven PCR chip. PCR reaction mixture is pushed around the circular channel by ferrofluid plug and flows through the three temperature zones continuously.

Fig. 2 Photograph of circular micro PCR device fabricated in PMMA by CO₂ laser ablation.

Fig. 4 (a) Schematic drawing showing temperature measuring points along the microchannel. (b) Temperature distribution in the PCR microchip. Data at each point was measured three times.

(a)

(b)

Fig. 5 Effect of cycle number on the efficiency of PCR. (a) UV image of PCR products with different cycle number. Lane 1: 100 bp DNA ladder, lane 2: PCR product with 10 cycles, lane 3: 15 cycles, lane 4: 20 cycles, lane 5: 25 cycles, lane 6: 30 cycles. (b) Relationship between band intensities and number of PCR cycles. Values were normalized with respect to the intensity obtained from the 500 bp fragment of the 100 bp ladder.

(a)

(b)

Fig. 6 Effect of cycle time on the efficiency of PCR. (a) UV image of PCR products at various extension times. Lane 1: 100 bp DNA ladder, lane 2: PCR product at extension time of 3 sec, lane 3: 6 sec, lane 4: 9 sec, lane 5: 12 sec, lane 6: 15 sec. (b) Relationship between band intensities and cycle time for microchip PCR. Values were normalized with respect to the intensity obtained from the 500 bp fragment of the 100 bp ladder.

Table 1. Conditions to perform PCR at various cycle times.

Extension time (sec)	Cycle time (sec)	Rotation speed of magnet (rpm)	Flow velocity of PCR reaction mixture (mm/s)	Complete time for 25 cycles (min)
3	9	6.67	7	3.75
6	18	3.33	3.49	7.5
9	27	2.22	2.32	11.25
12	36	1.67	1.74	15
15	45	1.33	1.4	18.75