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PAPER

An integrated PCR microfluidic chip incorporating aseptic electrochemical cell lysis and capillary electrophoresis amperometric DNA detection for rapid and quantitative genetic analysis†

Sandeep Kumar Jha,[a] Rohit Chand,[a] Dawoon Han,[a] You-Cheol Jang,[a] Gyu-Sik Ra,[a] Joung Sug Kim,[b] Baek-Hie Nahm[b] and Yong-Sang Kim*[ac]

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A fully integrated microchip for performing cell lysis, polymerase chain reaction (PCR) and quantitative analysis of DNA amplicons in a single step is described herein. The chip was built on glass substrate using an indium-tin-oxide (ITO) microheater and PDMS engraved microchannels, which integrated an electrochemical cell lysis zone, a continuous flow PCR module and capillary electrophoresis amperometric detection (CE-AD) system. The total length of the microchannel was 4625 mm for performing 25 cycles of flow-through PCR and was laid on a handheld form factor of 96 × 96 mm^2 area. The key to the fabrication of such a device lies in the use of a single medium to carry out different kinds of biochemical reactions and hence, a reagentless electrochemical cell lysis protocol was integrated on the microchip which was capable of lysing most cell types, including difficult to lyse gram positive bacteria. The lysate contained genomic DNA from a sample which was proven to be suitable for PCR reactions. Genetic analysis was successfully performed on the microchip with purified lambda phage genomic DNA and various cell types, including non-tumorigenic MCF-10A and tumorigenic MCF-7 human cell lines, gram negative bacteria *Escherichia coli* O157:H7, and gram positive bacteria *Bacillus subtilis*, at an optimized flow rate of 5 μl min^{-1}. For the detection of amplicon DNA, a CE-AD system was used, with semisolid alkaline agarose within the capillary microchannel to minimize interference from cell debris and for efficient resolution of DNA fragments. High signal to noise ratio during amperometric detection and the use of online FFT filtering protocol enhanced the limit of detection of DNA amplicons. Therefore, with a combination of portability, cost-effectiveness and performance, the proposed integrated PCR microchip can be used for one step genetic analysis of most of the cell types and will enable more accessible healthcare.

Introduction

The analysis of genetic materials in the post-human genome project era has become an ever-expanding branch of research and is thus routinely employed in the majority of biochemical laboratories. It has been used in a variety of applications such as genotyping, taxonomy, forensic investigation, cloning and molecular biology techniques, DNA sequencing, pathogen detection, drug discovery and environmental applications.[1] In a typical example involving DNA analysis, such as detection of a pathogen, the required steps include lysis of microbial cells, followed by polymerase chain reaction (PCR) for amplification of a specific stretch of microbial DNA and then analyzing the amplified DNA fragment by gel electrophoresis. The procedure usually completes with imaging the gel after electrophoresis followed by DNA elution and spectroscopic quantification of amplified DNA (amplicon). The entire process typically requires anything between half to a full day, and highly trained personnel to perform these specialized multiple analytical steps. In addition, these processes consume a lot of expensive reagents and require a number of specialized and often expensive instruments, such as a thermocycler and gel documentation system *etc.* Therefore, a critical need exists to miniaturize and automate such system. In this direction, a number of attempts have been made in the past to devise on-chip PCR systems, on-chip cell lysis systems and on-chip DNA detection platforms.[2–4] Most of these reports demonstrate the processes in isolation. A few scant attempts were made to integrate these processes into one integrated device, yet these efforts are still far from offering a reliable and complete solution, from DNA extraction to amplicon analysis.

[a]*Department of Nanoscience and Engineering, Myongji University, Yongin, Gyeonggi, Korea*
[b]*Division of Bioscience and Bioinformatics, Myongji University, Yongin, Gyeonggi, Korea*
[c]*Department of Electrical Engineering, Myongji University, Yongin, Gyeonggi, Korea. E-mail: kys@mju.ac.kr; Fax: +82-31-321-0271; Tel: +82-31-330-6365*
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We have extensively reviewed such reports whose analysis are presented below. The first micro-PCR chip was reported by Northrup *et al.*[2] and subsequently by various other groups.[3,4] However, most of these microchips, which have been described as successful, employed an optical method for detection of amplicon DNA. Considering that almost all of the samples used in PCR analysis are either turbid after DNA extraction or are colored with dyes, the resolution of optical detection is severely reduced. Though it has been standardized by researchers for a particular system, a wider approach is still desired so that the system can work under field conditions and with a wide variety of samples without interference. In this regard, we adopted an electrochemical detection method for DNA considering it to be free from interferences due to turbidity, viscosity and sample color.

Therefore, in the present work we fabricated a fully integrated microchip for performing cell lysis, PCR and analysis of DNA amplicons in a single step. The key to fabrication of such a device lies in the use of a single medium to carry out different kinds of biochemical reactions. For example, the buffer that is optimized for cell lysis should also suit the PCR conditions, as well as serve as a medium during analysis of amplified DNA. Keeping this in mind, we selected an electrochemical cell lysis procedure for effective and reagentless lysis of cells. The lysis of whole cells is a routine procedure in most biological laboratories, apart from being used as a source for pure DNA template in PCR analysis. The known methods for this purpose are high voltage electroporation;[5] proteinase-K, detergents and lysozyme treatment;[6] laser induced lysis;[7] bead milling and sonication;[8] freeze–thaw in liquid nitrogen, *etc.*[9] Various attempts in the past were made towards miniaturization of this technique for the development of an on-chip cell lysis device. However, their methods lacked the aim of miniaturization. For example, a few groups have used extremely high voltage sources (1–10 kV),[10,11] laser induced cell lysis,[12] or methods needing sample pretreatment with additional reagents.[13] On the contrary, the goal of the present study was to integrate a relatively inexpensive method for cell lysis that uses minimal reagents, power, and can be fabricated using common photolithography techniques. Also, the DNA to be obtained *via* this process should be aseptic and pure enough to be used in PCR without involvement of separation and purification stages. The principle used for cell lysis herein was based on our previous report of applying a DC voltage to electrochemically generate hydroxide ions inside the device. The hydroxide ions permanently hydrolyze the cell membrane, thereby releasing intracellular genetic material.[6,14] In continuation of this idea, the study was extended for efficient cell lysis of different cell types and optimum lysis conditions were evaluated.

The second module of the microchip was designed for PCR amplification procedure, which was carried out by continuously flowing the PCR mixture within a long microchannel laid over microfabricated indium-tin-oxide (ITO) microheaters. The devices were fabricated as a disposable system on glass substrate using polydimethylsiloxane (PDMS) polymeric material to hold microchannel design. PDMS is a widely used polymeric material in microfluidic devices because it can be easily fabricated by a negative molding method.[15] On the other hand, ITO heater electrodes were the choice for thermal cycling during PCR due to the ease of its fabrication and linear and rapid variation of its temperature by the application of DC power.[16]

The third module on the microchip was necessary for the separation and analysis of small DNA fragments obtained after PCR amplification. The analysis of DNA based on its size has long been a tedious and expensive routine in most of the molecular biology and forensic laboratories for genomic analysis, DNA fingerprinting, drug discovery, pathogen detection by PCR amplification and environmental analysis using conventional slab-gel electrophoresis techniques. In this process the separation matrix is formed by casting a thin slab of polymer gel material such as agarose. The agarose gel is known to provide a sieving action during electrophoresis to separate the analyte based on their molecular weight and is applicable to broad ranges of DNA fragment sizes. On the other hand, capillary electrophoresis (CE) is a powerful tool for the separation of DNA fragments in a narrow capillary under the influence of a strong electrical field. There are numerous advantages of using microchips for such applications, such as low consumption of samples and reagents, the possibility for on-chip sample mixing and handling, detection of analytes and products with high sensitivity and resolution, and elimination of laboratory apparatus. The application of agarose gel in a microchannel for CE operation can create different electrophoretic mobility according to the size of molecules and are separated in this process on the basis of its size to charge ratio. The electrical mobility of DNA during capillary electrophoresis has been shown to increase with decreasing molecular weight.[15] The apparent mobility of analytes such as DNA can be calculated from eqn (1):

$$\mu = \frac{L_d}{E \times t} \quad (1)$$

where, μ is the apparent mobility, L_d is the distance from the inlet to the detector (in cm), E is the electric field strength (in V cm^{-1}) and t is the time required for the sample to travel to the detector (in seconds).

After conventional capillary electrophoresis, the analytes are usually detected at the end of the capillary by amperometric or optical detection technique. However, it is quite difficult to miniaturize the optical detection method onto a microchip. The microchip based CE with amperometric detection (AD) is a feasible method for miniaturization and has been attempted in past for analysis of DNA restriction fragments and for sizing of PCR products.[15] There are numerous advantages of using on-chip AD over optical methods. The AD method does not require an expensive fabrication process and detection instrument assembly and does not have alignment issues as with optical detection, yet is capable of detecting analytes accurately and with short response time.[15]

Based on these facts and requirements, we finally integrated these modules on the same glass substrate for on-chip PCR analysis of a variety of target sequences including human genomic DNA marker for pancreatic/breast cancer. The tumor suppressor gene *SMAD-4* is amongst a few, which is widely reported to be missing in up to 50% individuals with pancreatic cancer.[17,18] Therefore, PCR based analysis of this gene in blood samples of individuals can lead to early prediction of occurrence of cancer and therefore increase the survival rate of cancer

patients due to better treatment. Apart from this, virus lambda phage, bacteria *E. coli* and *Bacillus subtilis* were also successfully analyzed using the integrated PCR microchip. The most significant improvement in such work was the ability of this microchip in reagentless lysis of most cell types and amplification of target DNA through PCR along with quantitative analysis of DNA amplicon size and concentration, all under an hour. Also, there were fundamental differences in the design of the microchip and its fabrication method when compared to a previous publication from our group.[19] In previous reports, we had used an additional thin membrane of PDMS to separate the ITO electrode of the microheater with that of the microchannel. It means that the ITO as well as the microchannels was on the same side of the glass substrate. Whereas, in the present design, we kept the ITO microheater on the back side of substrate, while keeping the Au microelectrode and microchannel on the same side of the glass chip. This ensured that the liquid was in direct contact with the Au electrode for cell lysis and because of this we could avoid the difference in plane between each module. Also, the liquid moving in the microchannel was in direct contact with hydrophilic glass all the time, thereby greatly reducing the shearing of DNA on the microchip, as seen in previous work. In our previous work, the presence of the hydrophobic surface throughout caused slight hindrance in liquid flow and resulted in elevated vapor pressure and erratic flow.

Moreover, our equipment happens to be the only report thus far to have cell lysis, PCR and quantitative electrochemical DNA detector on a single chip. Other groups have reported integration of either two modules with the exception of very few groups who have integrated all three modules, however their design and principle remains altogether different from ours, as they worked only for qualitative detection of DNA amplicons.[20–22] Also, those devices lacked sensitivity and required expensive and difficult to integrate optical components. Therefore, on a handheld form-factor, our equipment shall be closer to commercial realization owing to easy to fabricate and inexpensive components laid on a single chip.

Methods

Chemicals

High gelling temperature agarose (catalog number: A2929) and lambda phage DNA BstE II Digest (catalog number: D9793) having 1 $\mu g\ \mu l^{-1}$ DNA concentration (for 14 fragments total), out of which smallest fragment was 117 bp (40 ng μl^{-1} concentration) were purchased from Sigma Aldrich, Korea. All other chemicals were of analytical reagent grade and were used without purification.

Microchip fabrication

The microchip was developed over a glass substrate. The back side of this glass had a deposition of indium-tin-oxide (ITO). This layer was used to design a microheater for thermal heating applications due to its property of showing linear variation of its temperature by the application of DC power. It was patterned using conventional photolithography and wet etch processes.[23] For this purpose, positive photoresist (AZ1512, Micro-Chem Co.) was spin-coated on the ITO side of the glass (Samsung Corning) and then patterned using photolithography to cover the regions meant for ITO electrodes. The remaining ITO film was then etched using the $FeCl_3$/HCl solution for 2 h and photoresist was later removed.[23] Gold interdigitated microelectrodes to apply DC potential to the sample for cell lysis and the electrodes necessary for CE-AD were fabricated over the front side of glass by using photolithography and the evaporation method (Fig. S1, ESI†). A positive photoresist layer was first spin coated on the opposite side of cleaned ITO patterned glass wafer and exposed to UV light though a photomask containing electrode designs. Subsequently, a 50 nm Ti layer and 320 nm thick Au layer were deposited on this glass wafer using a vacuum thermal evaporator. Then, the patterned positive photoresist was removed by using an ultrasonic cleaner. In this manner, the interdigitated gold electrodes used in cell lysis – three electrodes for the electrochemical detection, namely working, reference and counter; an additional two electrodes for applying separation electric field; and three decoupler electrodes (for optional decoupling of separation electric field to the detection voltage) – were fabricated on a single glass wafer.

The microchannels used in this study were laid in PDMS polymer using negative molding method (Fig. S1, ESI†).[15] For this purpose, 120 μm thick negative photoresist (SU-8 2075, Micro Chem.) was spin-coated and patterned on a silicon wafer. A degassed mixture of Sylgard 184 silicone elastomer along with curing agent (in 10 : 1 v/v ratio) was poured on the SU-8 patterned wafer and cured for 4 h at 72 °C. The PDMS mold that formed (average 8 mm thickness) was peeled off manually and drilled to make access holes of 3 mm diameter each. The width and depth of the microchannel created were 250 μm and 120 μm respectively. For finalizing the device fabrication steps, the PDMS mold and glass substrate containing ITO/gold electrodes were bonded with each other by UV-ozone treatment, while keeping the gold side of the glass in contact with PDMS. ITO heaters were calibrated by inserting thermocouples into the microchannel and flowing deionized water through it. Fig. S1 (ESI†) shows the schematics of the fabrication process of continuous-flow PCR chip, while Fig. 1 illustrates the integrated microchip.

Cultivation of cells

The human cell line MCF-7 was cultivated in DMEM (Dulbecco's Modified Eagle Media, Gibco) supplemented with 10% fetal bovine serum (Gibco), 0.01 mg ml^{-1} bovine insulin (Sigma) and 100 units/ml penicillin–streptomycin (Gibco) to 70% confluence as per the method described by Kim *et al.*[24] The cells were then harvested by trypsinization with 0.05% Trypsin and 0.53 mM EDTA. The detached cells were centrifuged, resuspended (to 2×10^7 cells ml^{-1} concentration) and kept in pH 7.4 PBS at 4 °C until cell lysis. The MCF-10A cells were cultured in MEGM (Mammary Epithelial Growth Medium, Serum-free, Clonetics) supplemented with 100 ng ml^{-1} cholera toxin (Sigma) to 70% confluence as per the method described by Caldas *et al.*[25] The detached cells were centrifuged, resuspended (to 2×10^7 cells ml^{-1} concentration) and kept in pH 7.4 PBS at 4 °C until cell lysis. *Escherichia coli* O157 (EDL 933) serotype O157:H7 strains were grown on 5% sheep blood agar plates (Becton, Dickinson and Company, Sparks, MD) at 37 °C for 24 h. Prior

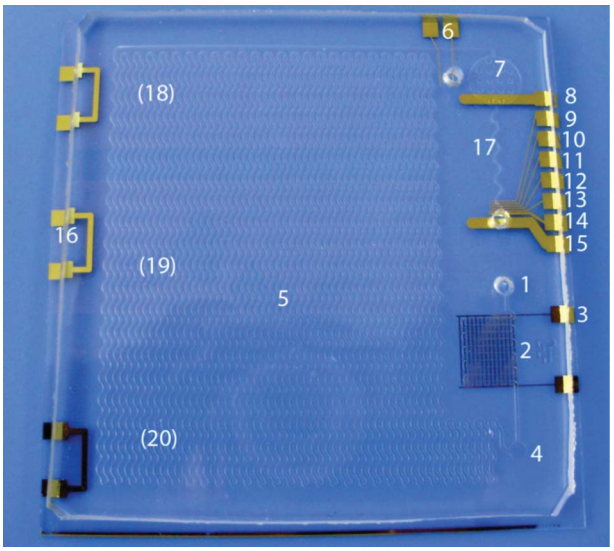

Fig. 1 The PCR microdevice showing glass substrate, ITO microheaters, PDMS mold containing microchannels, gold microelectrodes for cell lysis, PCR amplification and CE-AD operation. (1) inlet reservoir for applying sample onto the microchip; (2) Gold interdigitated microelectrodes and (3) contact pad for applying DC potential for electrochemical cell-lysis; (4) reservoir for manual extraction of cell lysate from the chip for conventional analysis; (5) microchannels for carrying out 25 cycles of PCR; (6) gold electrodes used for conductometric liquid level sensor used as circuit breaker for syringe pump so that sample flow can be stopped once large reservoir is completely filled with PCR amplicon sample; (7) large reservoir for collecting PCR amplicon sample; (8) and (15) gold electrodes used for applying separation voltage for CE operation; (9–11) optional decoupler electrodes; (12) reference electrode; (13) working electrode; (14) counter electrode; (16) optional resistance temperature detector for feedback temperature control of ITO microheater; (17) spiral CE- microchannel filled with semisolid agarose dissolved in NaOH medium for CE-AD separation of PCR amplicon; (18–20) ITO microheaters on the back side of glass as thermocycler zones for PCR, namely: extension, annealing and denaturation.

to cell lysis, bacteria were grown in 10 mL Penassay broth (Becton, Dickinson Co.) overnight at 37 °C. An overnight culture (1×10^9 CFU per mL) of *Escherichia coli* O157:H7 was used to inoculate 10 mL of sterile Penassay broth (6 h re-growth in 37 °C).[26] The bacterial cells were harvested, re-suspended in PBS to approximately 2×10^7 cells ml^{-1} before being used for cell lysis. *Bacillus subtilis* DSM 5750 strain was grown aerobically in 10 ml nutrient broth at 30 °C under shaking condition at 200 rpm.[27] The cells were harvested, resuspended in PBS to approximately 2×10^7 cells ml^{-1} before being used for cell lysis.

Cell lysis

A suspension of 50 μl (10^6 cells) cells from either cell types (human cell line MCF-10A or MCF-7 or bacteria *Escherichia coli*) in PBS was injected into the silicone tube attached with the microchannel using the precision syringe pump (KDS100, KD Scientific). On the other hand, it was difficult to lyse gram positive bacteria *Bacillus subtilis* using just PBS as suspension medium. Therefore, 0.1% or 2% tween-20 was added in the PBS and between 0–10 V of DC bias was applied for lysis. For this, 15 ml of nutrient broth grown cells (for 5 h) was harvested and the pellet was divided into 3 parts (150 μL each). The 1st part was suspended in PBS, the 2nd in 0.1% tween 20 in PBS and the 3rd part in 2% Tween 20 in PBS solution. The lysate was collected through a reservoir made at the end of the cell lysis module (Fig. 1, item 4) and used in spectroscopy and agarose gel electrophoresis for the confirmation of DNA release.[14] After lysis, the cell lysate was analyzed for the presence of released genomic DNA using conventional 1% agarose gel electrophoresis. After electrophoretic separation, the genomic DNA band was cut with a sterile knife and re-dissolved in Mega Spin™ gel elution kit (Intron Biotech., Korea) for spectroscopic analysis at 280 nm wavelength using an Eppendorf Biophotometer. For comparison, genomic DNA of lambda phage viral DNA (1.8 μg, suspended in 50 μl PBS) (Takara, Shiga, Japan) was also subjected to similar treatment and the liquid collected after passing over cell lysis module was analyzed electrophoretically and spectroscopically.

On-chip PCR

In order to ascertain PCR readiness of the extract collected from cell lysis module, the mixture was subjected to conventional PCR (Applied Biosystems Thermal cycler, model 2720). The cell sample for human cell line MCF-10A was analyzed for the presence of *SMAD4* gene. Similarly, for comparison, the mixture corresponding to genomic DNA of lambda phage viral DNA, as collected from cell lysis module was also analyzed with conventional thermocycler.

Subsequently, the fabricated device was used for on-chip PCR involving the mixture obtained through on-chip cell lysis. For this purpose, approximately 10^6 cells of each cell type were suspended in 50 μl PBS and 60 μl 2× PCR mix (Intron Biotechnology, Korea) containing Taq-DNA polymerase and 5 μl each of the primers (1 pmole/μl each) against the target gene, as described in Table 1, were mixed with it. The mixture was injected into microchannel through the silicone tube with the help of a precision syringe pump.

Temperature control is the one of the most critical factors in PCR. In order to perform PCR successfully, the temperature should be maintained at a constant level in each temperature zone. To measure the temperature of the glass over which the PCR mixture flows, three thermocouples were inserted between the glass and the PDMS block for calibration of temperature (thermocouples were not used in the final chip design). The on-chip PCR was initiated with different on-chip temperature zones of 92, 55 and 72 °C for melting, annealing and extension of DNA, respectively, for 25 cycles. The PCR product was collected at the end of the microchannel leading to a large reservoir (8.75 μl volume) (item no. 6, Fig. 1) and the amplicon DNA was verified by 1% agarose gel electrophoresis (with ethidium bromide) as well as spectroscopic method.

CE-AD operation

The sample collected in the large reservoir leading to CE-AD microchannel was subjected to CE-AD analysis. Prior to this, the method for CE-AD of DNA was perfected through a series of experiments. The DNA molecule is composed of adenosine and guanosine purine nucleosides which are electrochemically active.[15] Therefore, we first analyzed their redox peak voltages

Table 1 Summary of the primers used for PCR involving different cell types

Cell type	Target gene	Forward primer	Reverse primer	Amplicon size	PCR annealing temperature
Human cell line MCF10A	*SMAD4*	GTCTATGGCACATCAAA-CTATGCACAATGC	GTCTAACAATTTTCCTTGCAACG	193 bp	55 °C
Human cell line MCF-7	*SMAD4*	GTCTATGGCACATCAAAC-TATGCACAATGC	GTCTAACAATTTTCCTTGCAACG	193 bp	55 °C
Escherichia coli O157:H7 EDL933	SSU r-RNA	GAACGGTAACAGGAAGAA	ATTACCGCGGCTGCTGGC	471 bp	55 °C
B. subtilis	16S r-RNA	AAGTCGAGCGGACAGATGG	CCAGTTTCCAATGACCCTCCCC	595 bp	55 °C
λ-Phage	Genomic DNA	GCA-AGT-ATC-GTT-TCC-ACC-GT	TTA-TAA-GTC-TAA-TGA-AGA-CAA-ATC-CC	100 bp	55 °C

through conventional cyclic voltammetry. The conventional cyclic voltammetric (CV) studies were carried out with a three-electrode system and a CH instruments electrochemical analyzer (CHI 800B from CH instruments, USA). The electrochemical experiments were performed in 100 mM NaOH solution with 100 mM adenosine and guanosine (Sigma Aldrich) using conventional (gold working, Pt counter and Ag/AgCl reference) electrodes from CH Instruments. NaOH was used as media as well as electrolyte because guanosine molecules have poor solubility in water. The potential was cycled from +1.5 V to −0.5 V with a scan rate of 100 mVs^{-1}. Through this CV experiment, we could find the detection voltage to be applied in capillary electrophoresis amperometric detection (CE-AD) module and peak current range that the chemicals would generate. However, a semisolid medium was required for the CE-module to counter the hydrodynamic pressure of liquid moving out of PCR module, otherwise CE-AD analysis could be severely affected. Therefore, we added 1% agarose prepared in 100 mM NaOH. The CE-AD microchannel of 5 cm effective length was filled carefully with this agarose medium through reservoir (placed over item no. 15 on Fig. 1).

During calibration of the CE-AD process, a small hole was drilled into the large reservoir and it was filled with gel. Then, a 1 μl testing sample was introduced into this reservoir using a micropipette. After the sample loading, an electric field of 100 V was applied between the inlet reservoir and the waste reservoir (items 8 and 15, Fig. 1) to initiate electrophoretic separation. Amperometric detection in the CE-AD microchip was performed with three-electrode configuration (items 12–14, Fig. 1) placed in the path of analyte flow. The detection voltage was set as +0.8 V. Redox reaction of adenosine or guanosine from testing analytes on the working electrode generated current peaks, which was detected, recorded and stored directly on a computer using an electrochemical analyzer. Similar conditions were maintained for DNA test samples. Later, during on-chip simultaneous cell lysis, PCR and CE-AD, the large reservoir was not punctured through the top, rather the sample arriving from PCR module was diverted to adjacent reservoir equipped with an on-chip conductivity sensor (item 6, Fig. 1). The conductivity sensor was interfaced with an op-amp circuit and ADC card and its role was as a circuit breaker for syringe pump. This means, when the large reservoir leading to CE-AD microchannel was completely filled, the excess solution would short the conductivity sensor, and the flow of liquid will stop automatically. The CE-AD operation was initiated approximately 5 min after this event, so as to minimize any remaining hydrodynamic flow of the sample.

Microchip operation

The overall experimental procedure was carried out using the following pathway. The CE-AD microchannel (item 17, Fig. 1) was first filled with 1% alkaline agarose gel and left for solidification. Later a calibrated DC bias was applied to the ITO electrodes laid on the back side of the glass substrate for heating the denaturation (20), annealing (19) and extension (18) PCR zones (Fig. 1). To start the analysis, a low DC voltage was applied to the interdigitated gold electrode (item 2, Fig. 1) and a mixture of cells suspended in PBS buffer and pre-PCR mix containing dNTPs, primers, DNA polymerase, reaction buffer, and $MgCl_2$ (120 μl total volume) was injected into the inlet (reservoir 1, Fig. 1) through a silicon tube using a syringe pump. The cells flowing in the microchannel underwent lysis due hydroxyl ion generation but the genetic material and pre-PCR mix remained intact. After lysis, the sample entered the PCR zone (region 5, Fig. 1). At this zone, the amplification of the target gene took place with the help of the enzyme and cyclic change in temperature. Finally, after amplification, the amplicon entered the large reservoir (item7, Fig. 1) and the remaining sample was allowed to pass to the exit reservoir, having gold conductometric liquid level sensor electrodes (item 6, Fig. 1) (connected with an ADC card, model 6013 from National Instruments, USA, through in-house built op-amp circuit) that were used as circuit breakers for the syringe pump so that sample flow can be stopped once the large reservoir is completely filled with the PCR amplicon sample. Finally, a separation voltage was applied to the separation electrodes (items 8 and 15, Fig. 1) and the DNA samples passed through a spiral microchannel (item 17, Fig. 1) filled with alkaline agarose semisolid medium. The sample entered the CE microchannel and got separated based on size and charge ratio. The DNA amplicons were finally detected using a three-electrode electrochemical detector with reference, working and counter electrodes (item 12–14 respectively, Fig. 1). The syringe pump, external power sources for application of CE-separation voltage and cell lysis and the CE-AD detection stages were controlled using software written with LabVIEW code. The left over amplified product could be drawn using a syringe for further analysis.

Results and discussion

Microchip design

The microchip was fabricated by standard photolithographic procedures on a glass substrate, where microheaters made up of an ITO layer was fabricated on the back side of the same glass.

Microchannels were engraved in PDMS layer by negative molding method from a SU-8 pattern designed on a Si wafer. The microchannel layer and glass were bonded using UV-ozone cleaner. The integrated chip had a total area of 96 × 96 mm^2. Such a dimension of the microchip was suitable enough to develop into a hand-held device. The microchip contained three zones: cell lysis, PCR and CE-AD. The microchannel in the cell lysis region had a length of 115 mm, followed by 4510 mm in the PCR module for 25 cycles of PCR (Fig. 1). The PCR module also contained provision for pre-PCR denaturation (92 °C) and final extension (72 °C). The ratio of the channel lengths of the three different temperature zones for PCR, denaturation (92 °C), annealing (55 °C), and extension (72 °C), was 2 : 2 : 3 and was placed in this sequence, separated by a calibrated gap to ensure cooling of circulating liquid in between the temperature zones. The longitudinal gap between 92 °C ITO and 55 °C ITO was kept at 19.67 mm compared to 6 mm between 55 °C ITO and 72 °C ITO at a ratio of 77 : 23, keeping in mind the thermal conductivity constant (k) of Sylgard PDMS as 0.16 W/mK, which means the liquid would dissipate enough heat in between the different temperature zones and can equilibrate quickly into the new zone. The temperatures necessary to carry out PCR were provided by applying a DC potential to the underlying patterned ITO microheaters. The voltage dependence of ITO temperature is a well-established fact and was calibrated under our conditions at 5 μl min^{-1} flow rate of deionized water (DIW) into the microchannel. The power requirements *versus* obtained temperature for three thermal zones are shown in Table S1, ESI.† The retention time for the PCR pre-mix flowing in the microchannel at 5 μl min^{-1} rate during cell lysis and PCR stages was 22.2 min. The CE-AD module had 5 cm long microchannel for electrophoretic separation of analytes. The electrochemical detection of samples was performed by using on chip microelectrodes.

Cell lysis

In addition to the optimized design parameters for maintaining stringent temperature control and flow, it was also a challenge for us to maintain a single medium for cell lysis, PCR and CE-AD operation. The medium or buffers usually used for cell lysis interfere with PCR operation. The PCR mixtures often have low conductivity for a CE operation and so on. This was the reason why we chose electrochemical cell lysis as a mode to obtain PCR grade template DNA. The PCR mixture could be directly applied for cell lysis without affecting the catalytic activity of this mixture. However, for CE-AD operation, a strong basic medium was necessary. We used agarose semisolid medium prepared in NaOH solution to carry out CE-AD while eliminating the hydrodynamic pressure of the flowing PCR mixture.

A number of reports on a chip based PCR had previously described the phenomena of adherence of the sample and proteinic components on the walls of PDMS microchannel, thereby limiting the ability of microchip to perform PCR. Hence, a lot of modification processes were required for such chips before being able to perform PCR. One of the interesting solutions for this was to use bovine serum albumin or run a template-less PCR mixture into the microchannel first.[28] In our case, we eliminated such a problem by combining the cell lysis with PCR module. The cell debris generated as a result of lysis travel within the microchannel and adheres to the walls. This somehow leaves the template DNA available for the reaction. Therefore cell lysis had a major influence on the success of this microchip. A limited trial of this process in conjugation with on-chip PCR was conducted by our group in the past, leading to the success of the cell lysis process.[19] The study was limited to human cell line cells, which are the easier target for lysis in the absence of additional reagents. We extended such study in the present work to include bacterial cells, which also included gram positive bacteria which are known to be more difficult to lyse, even after using numerous reagents, including detergents, lysozyme *etc.* Our aim was to restrict the use of such chemicals which may otherwise hinder the PCR process upstream.

Under this cell lysis protocol, common cell types including gram negative and gram positive bacteria, and animal cells including human cell lines MCF-10A and MCF-7, were subjected to lysis on our microchip platform. The electrochemical lysis of the human cell line could be achieved between 2 V and 5 V of DC input with the optimum release of genomic DNA at 5 V for 5 min.[14] The alternate +5 and 0 V DC across 200 μm gapped Au interdigitated electrode configuration helped in efficient cell lysis without the use of additional reagents. The process generated hydroxyl ions desired for lysis and at the same time, electrode impedance was low enough to avoid generation of Joule heat inside the microchannel.[29] Therefore, this method was supposed to yield cellular components in their native state. It also reduced dependence on microchannel preconditioning as used by various other groups[22,30] to minimize the adherence of template DNA as well as PCR amplicon to the side walls of the microchannel. Since PBS buffer was used as a carrier solvent, inhibitory components for PCR such as SDS (>0.005%), phenol (>0.2%), ethanol and isopropanol (>1%), sodium acetate (>5 mM), NaCl (>25 mM), EDTA (>0.5 mM), *etc.* as used inadvertently by various groups during cell lysis steps,[22] could be eliminated. The bright field microscopic images of MCF-7 cells have been shown in Fig. 2a (before lysis) and 2b (after lysis), which illustrated complete lysis of cells within 3 min of application of 5 V DC bias. The cell lysate was studied for the presence of genomic DNA from MCF-7 cells through agarose gel electrophoresis (0.7% agarose in TBE). As can be seen in Fig. 2 (c), the optimum release of genomic DNA from the cells was when 2 V or more of DC potential was applied (can be visualized near the sample well). The same range of voltage was applicable for lysis of gram negative bacteria *Escherichia coli* (Fig. 2d, band can be located above the 10 kB marker). However, it was difficult to lyse gram positive *Bacillus subtilis* cells. Therefore Tween 20 was added to the lysis mixture, considering that it in low concentration does not adversely affect PCR.[31] The gel image obtained with cell lysate of *B. subtilis* showed optimum release of its genomic DNA by application of 5 V DC onwards in the presence of 0.1% Tween 20 (Fig. 2e, band can be located above the 10 kB marker). These results suggest that the electrochemical cell lysis method was an efficient and convenient technique suitable for lab-on-a-chip systems and can be practically used in the lysis of almost any cell-types. The cell lysis released genomic DNA and other genetic materials from the cell which can be used for PCR on the microchip in aseptic conditions, if needed.

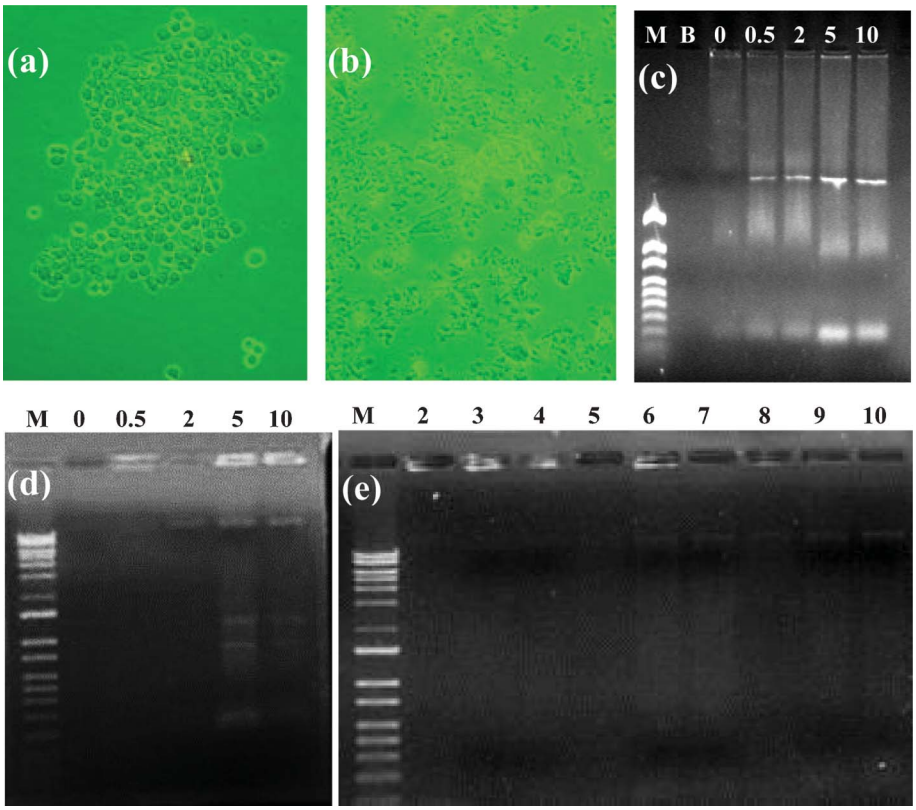

Fig. 2 Unified cell lysis protocol for all common types of cells including bacteria and animal cells. MCF-7 human cell line cells before (a) and after (b) application of 5 V DC bias on the microchip for 3 min (as seen through a bright field microscope when samples were placed on a glass slide and covered with glass cover slip). The cell lysate was studied for the presence of genomic DNA from MCF-7 cells through agarose gel electrophoresis (0.7% agarose in Tris-borate EDTA buffer). (c) Optimum release of genomic DNA from the cells could be seen (near the well) when 5 and 10 V of DC was applied. In this figure, M = 1 kb DNA ladder marker (6 μl sample loading on the gel), B = cell lysis reaction without any cells (blank) and other values represent DC voltages applied (all 20 μL loading). (d) The gel doc obtained for cell lysis of gram negative bacteria *Escherichia coli*. M = 10 kb marker (6 μl sample loading on the gel) and other figures represent DC voltage values as applied onto cell lysis module with PBS buffer (all 20 μL loading). Optimum DNA release can be seen by application of 2 V DC onwards (band can be located above 10 kb marker) (e) *Bacillus subtilis* (Gram positive) lysis. Lanes: M = 10 kb marker (6 μl sample loading on the gel, others 20 μl loading); 2 = 0 V, 3 = 5 V, 4 = 10 V in only PBS; 5 = 0 V, 6 = 5 V, 7 = 10 V in 0.1% Tween 20 and PBS; 8 = 0 V, 9 = 5 V, 10 = 10 V in 2% Tween 20 and PBS. Optimum DNA release can be seen by application of 5 V DC onwards in presence of a minimum of 0.1% Tween-20 (band can be located above 10 kb marker).

On-chip PCR. The cell lysate for MCF-10A cell line from lysis module was first subjected to conventional PCR analysis. The primer set used was for amplifying *SMAD4* gene for 25 cycles of PCR using conventional thermocycler. The DNA amplicon obtained in this way was verified with conventional slab gel electrophoresis for presence of desired 193 bp product (Fig. 3a). This confirmed the PCR readiness of extracted DNA through on-chip cell lysis.

Other concerns for the proposed device were to ascertain the effect of cell lysate on the PCR reaction and to optimize the flow rate of sample within the microchannel, as it can influence the kinetics of the enzymatic reaction by controlling the residence time of a fluid in a specific temperature zone. For this reason, a sample solution containing purified genomic DNA of lambda phage virus (which was not expected to generate cell debris) along with PCR pre-mix was passed through cell lysis and PCR modules at different flow rates. Fig. 3(b) shows the PCR product band (through agarose gel electrophoresis) *versus* flow rates. A flow rate of 5 μl min^{-1} showed a good amplified band (lane 4) compared to other flow rates. Non amplification of template

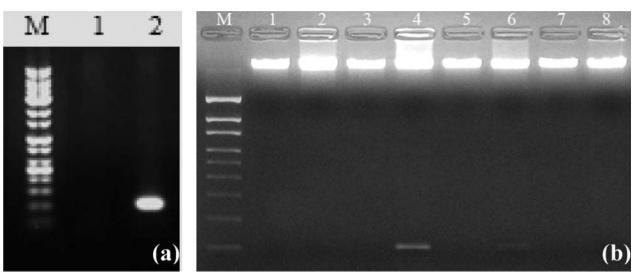

Fig. 3 (a) Gel doc for conventional PCR with MCF-10A cells for *SMAD4* gene (5 μl loading in each well): lane M = 10 kb marker; 1 = control; 2 = sample; (b) On-chip PCR analysis of purified λ-phage genomic DNA: effect of flow rate on on-chip PCR amplification. Lane: M = 1.5 kb marker; 1 = Pre PCR mixture for 1 μl min^{-1} flow rate; 2 = PCR at 1 μl min^{-1} flow rate; 3 = Pre PCR mixture for 5 μl min^{-1} flow rate; 4 = PCR at 5 μl min^{-1} flow rate; 5 = Pre PCR mixture for 10 μl min^{-1} flow rate; 6 = PCR at 10 μl min^{-1} flow rate; 7 = Pre PCR mixture for 25 μl min^{-1} flow rate; 8 = PCR at 25 μl min^{-1} flow rate (5 μl marker and 20 μl loading in each well).

DNA is attributed to the low or excess heating in other flow rates leading to de-activation of enzymes. The liquid flowing through microchannel thus had a retention time of about 30 s in each zone at a flow rate of 5 μl min^{-1}. The benefit of having a flow-through microfluidic chip for PCR amplification as compared to single drop static amplification models proposed by other groups[22,30] is inefficient control of the temperature for PCR.

The fabricated device was subsequently used for on-chip PCR amplification of *SMAD4* gene from the human cell lines. The cell suspension of non-tumorigenic MCF-10A or tumorigenic MCF-7 human cell-line mixed with PCR mixture containing primers for tumour suppressor gene *SMAD4*[32] was allowed to flow through cell lysis and PCR modules in succession for 25 cycles of amplification. The conventional agarose gel electrophoresis of reaction product verified the presence of 193 bp amplicon from the on-chip PCR reaction for MCF-10A cell lines (Fig. 5b, lane B5), whereas no product was obtained for MCF7 sample, which was expected as the *SMAD4* gene is absent in MCF7 cells. This confirmed the feasibility of the developed microchip for PCR amplification of genetic materials and also disproved the notion that cell debris could inhibit the on-chip PCR reaction. The amplicon yield was approximately 250 ng DNA/10^6 MCF-10A cells, as also verified by agarose gel electrophoresis (Fig. 5b, Lane B5). The result also indicates that the proposed device can be used for early prediction of cancer by analysing tumor suppressor genes such as *SMAD4*. The device can also be used in almost any other genetic analysis involving DNA extraction and PCR amplification in a rapid and cost-effective manner.

Therefore, in order to prove the conceived idea, we further performed on-chip cell lysis to PCR amplicon detection of purified λ-phage genomic DNA, bacteria *Escherichia coli* O157:H7 EDL933 and *B. subtilis* in the PCR microchip (at optimized flow rate of 5 μl min^{-1}). As can be seen in Fig. 5a, the on-chip cell lysis combined with PCR produced the desired results. The PCR amplification of purified λ-phage genomic DNA produced a detectable amount of DNA amplicon of 100 bp size after 25 reaction cycles (Fig. 5a, lane A3). These amplicons were collected from the large reservoir (Fig. 1, item 6) for verification by conventional gel electrophoresis. The on-chip PCR of gram negative bacteria *E. coli* produced 471 base pair amplicon (Fig. 5c, band on lane C3), while more difficult to lyse gram positive *B. subtilis* cells produced the desired 595 base pair band on lane D3, Fig. 5d. The sample collected in the collection reservoir on the microchip, (Fig. 1, item 7) was subjected to subsequent CE-AD by the application of DC bias between electrodes 8 and 15 (Fig. 1), while the curved capillary (item 17, Fig. 1) used for CE-separation was filled with agarose gel as a sieving medium.

On-chip CE-AD analysis of DNA and PCR amplicons. The CE-AD analysis of DNA worked on the principle of amperometric detection of guanosine base of DNA over the in-channel gold three-electrode electrochemical system. Prior to the analysis of PCR amplicon, the method for CE-AD of DNA was perfected through a series of experiments, including electrochemical studies of adenosine and guanosine purine nucleosides. The conventional cyclic voltammetric (CV) studies in 100 mM NaOH solution with 100 mM adenosine and guanosine showed a well defined oxidation peak at 0.82 V corresponding to guanosine and 1.1 V for adenosine (Fig. S3, ESI†).[15] No reduction peak was observed in the cathodic sweep, thus suggesting that electrochemical oxidation of adenosine and guanosine are irreversible processes. Therefore, we applied 0.82 V as the detection voltage (corresponding to guanosine) during all CE-AD measurements for detection of DNA fragments and PCR amplicons and recorded the redox current peaks for guanosine from testing analytes. For capillary migration of negatively charged DNA molecules, a constant voltage of 100 V DC was applied between separation electrodes (items 8 and 15, Fig. 1). Higher separation voltage was avoided as it led to electrode corrosion and Joule heating within the microchannel. It is common to use in-channel decoupler electrodes for such CE-AD measurements, in order to reduce noise in the detection signal. The role of the decoupler is to nullify channel current due to electrical separation voltage. However, this method severely dents the performance of CE-separation, for the reason that the separating molecules retard near decoupling electrodes due to voltage drop. The journey of these molecules from this junction towards detection electrodes are thus driven mainly by diffusion. However, by removing the decoupler electrodes altogether, the amperometric detection becomes more tedious due to the presence of noise in the detection signal. This problem was obviated in our present study by using a relatively new technique of real-time FFT filter to smoothen peak definitions obtained from CE-AD. This approach greatly reduced noise in the detection signal and improved the rate of identification of peaks in correspondence with size of molecules in complex mixtures such as DNA ladder.

As a DNA test sample, the CE-AD was first conducted with *BstE* II digest of Lambda-phage DNA. For CE-AD of analyte, 1 μl of the sample was injected into the sample reservoir using a micropipette and an electric field of 100 V was applied between the inlet reservoir and the waste reservoir. Fig. 4a and Fig. S4a,b (ESI†) show the electropherograms of lambda phage DNA digest. This result was used to calculate the migration time of different length of DNA fragments and the level of current produced by a particular concentration of DNA. A typical sigmoidal correlation between DNA concentration and response current was obtained (inset of Fig. 4b) which can be used as a standard for detecting unknown samples later on. The linear range for this detection was around 20–700 ng with a sensitivity of 0.149 μA ng^{-1} DNA. The LOD for this method was 1 ng of DNA (Fig. S6, ESI†). The detection (migration) time was dependent on size of DNA fragment. The migration time of DNA fragments could be calculated using eqn (1) and Fig. 4b and latter simplified into eqn (2) after curve fitting in Origin software.

$$\text{DNA length(base pair)} = 34.614\left(\frac{-122.39991}{698.01561-\text{migration time(sec)}}\right)^{-2.06673} - 34.614 \quad (2)$$

The concentration of DNA was calculated using inset data to Fig. 4b and upon curve fitting using Hill1 function in Origin software, it can be simplified as:

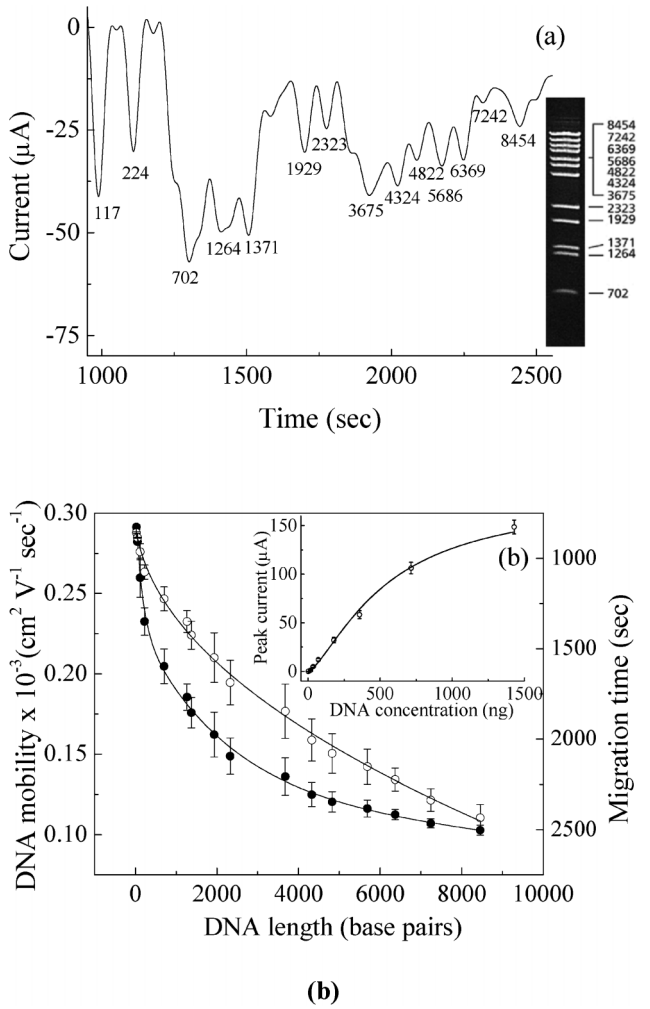

Fig. 4 CE-AD of Bst E-II digest of genomic DNA of lambda phage: (a) Electropherogram with FFT filtering (inset shows gel doc of same sample), (b) mobility of DNA (–●–) and DNA migration time (–○–) for different lengths of DNA fragments, (inset to b) concentration of DNA sample *vs.* averaged response peak (calculated for 117 bp peak).

$$\text{DNA concentration(ng)} = 543.0898\left(\frac{0.01004+\text{response current}(\mu\text{A})}{179.75646-\text{response current}(\mu\text{A})}\right)^{0.70502} \quad (3)$$

Using these standardizations, the on chip PCR product was finally detected using the CE-AD module of our integrated device. In a similar pattern, the analytes entered the microchannel and underwent electrophoretic separation due to the presence of electric field. Fig. 5e shows the electropherogram of different analytes after on chip polymerase chain amplification. The length and the concentration of amplicon were calculated (Table 2) from eqn (2) and (3) above. These detections were quite sensitive and reproducible owing to the high signal to noise ratio of CE-AD due to online FFT filtering protocol (Fig. S6, ESI†). The peak detection currents for each amplicon also fell well within the linear detection range and detection sensitivity of the device and hence the integrated PCR microchip can be used for

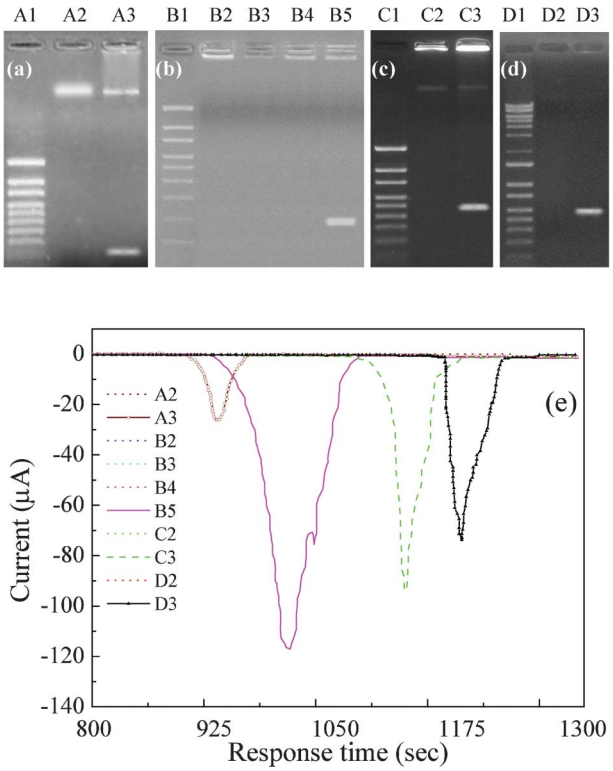

Fig. 5 (a) On-chip PCR analysis of purified λ-phage genomic DNA, lanes: A1 = 1.5 kb marker, A2 = control sample without the addition of primer and A3 = sample with primer (100 base pair band on lane A3 is the desired PCR product after 25 reaction cycles); (b) on-chip PCR analysis of MCF-7 and MCF-10A cells (*SMAD4* gene), lanes: B1 = 1.5 kb marker; B2 = Pre PCR lysate for MCF-7; B3 = PCR of MCF-7; B4 = Pre PCR lysate for MCF-10A (Non-cancerous); B5 = PCR of MCF-10A (the 193 base pair band on lane B5 is the desired PCR product after 25 reaction cycles); (c) On-chip PCR analysis of *E. coli* DNA, lanes: C1 = 1.5 kb marker, C2 = control sample without the addition of primer and C3 = sample with primer (the 471 base pair band on lane C3 is the desired PCR product after 25 reaction cycles); (d) On-chip PCR analysis of *B. subtilis* cells, lanes: D1 = 1.5 kb marker, D2 = control sample without the addition of primer and D3 = sample with primer (the 595 base pair band on lane C3 is the desired PCR product after 25 reaction cycles); flow rate in all reactions: 5 μl min^{-1}; (e) response current *versus* detection time for concurrent on-chip CE-AD of DNA amplicons for samples (A–D) above.

one step genetic analysis of most of the cell types, as experimentally proven with the present set of results.

Demerits of proposed device. One of the demerits of this lab-on-a-chip was that it suffered the same fate of unspecific PCR if the temperature was not maintained properly, as in the case of conventional PCR. Therefore, a 30 min microheater equilibration period was maintained before loading the sample onto the microchip.

Another demerit of our microchip is that the total time required from the point of application of the sample until the detection of DNA is still over 40 min (22 min for cell lysis to PCR and 15–20 min to detect PCR amplicon, as tested in present work), which can be considered a shortcoming for a handheld point-of-care device. However, there is a fair bit of scientific

Table 2 On-chip PCR amplification of different cells and DNA types and their subsequent CE-AD response

Sample name	Expected amplicon DNA size (bp)	Detection time (sec)	Amplicon quantity in the wells of electrophoresis gel (20 μl loading) (ng)	Amplicon quantity in large reservoir (35 μl) (ng)	Response current (δμA)
λ-phage genomic DNA	100	938	80	140	26.3
MCF-10A human cell line	193	1020	485	848.75	117
Bacteria *E. coli* cells	471	1151	360	630	93.8
Bacteria *B. subtilis* cells	595	1213	250	437.5	73

challenge that remains, especially for the way PCR technology works. The reactants need to remain at a particular temperature for a minimum of a few seconds and solution should cool down before flowing to another temperature zone. Besides, there should be a pre-denaturation and final extension cycles long enough to affect the reaction. Also, by reducing the number of cycles below 25 leads to lower detection sensitivity. Therefore, to overcome these challenges, we attempted to increase the flow rate of liquid by using fast acting Herculase DNA polymerase, but that did not improve the total reaction time much. As Taq polymerase is much cheaper compared to Herculase, we would recommend sticking to the former for commercial success, while sacrificing a few minutes of reaction time. Another demerit of our device is a lack of on-chip micropump, which is crucial for realization of a handheld device. However, it still remains a distant possibility, as no existing micropump has the ability of pumping liquid for a prolonged period of time, especially with PDMS architecture. We are currently in the process of trying to develop such long duration micropumps[33] for these applications and will then attempt to integrate them with our present device.

Merits of the proposed device. Though other groups have reported integration of two to three required modules for on-chip PCR, their design and principle remains altogether different from ours and they mainly worked for qualitative detection of DNA amplicons.[20–22] Their method lacked sensitivity and required expensive and difficult to integrate optical components, whereas our microchip could quantitatively analyse DNA amplicons on a single analysis. Moreover, optical detection of PCR amplicon has its flaws as the cell debris would also be optically sensitive and may provide false results. Our use of polymer gel for CE-AD removes such possibility of interferences, as the chances of cell debris and proteins entering the gel and reaching the detector before the DNA fragments are very remote. Therefore, we can conclude that our device is another step closer to realization of a fully functional lab-on-a-chip, minus the micropump that has the capability of highly accurate quantitation of PCR amplicons.

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