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Research Article

Capillary electrophoresis microchip for direct amperometric detection of DNA fragments

Detection and quantitation of nucleic acids have gained much importance in the last couple of decades, especially in the post-human genome project era. Such processes are tedious, time consuming and require expensive reagents and equipment. Therefore, in the present study, we demonstrated a simple process for the separation and analysis of small DNA fragments using capillary electrophoretic amperometric detection on an inexpensive disposable glass microchip. The device used polydimethylsiloxane engraved microchannel and Au/Ti in-channel microelectrodes for sample detection. The DNA fragments were separated under low electric field (20 V/cm) for improved detection sensitivity and to retain the biomolecules in their native conformation. With a low sample requirement (as low as 1 μL) and high reproducibility, the proposed microchip device was successful in resolution and detection of DNA fragments of various lengths.

Keywords:
Amperometric detection / Capillary electrophoresis / CE-AD microchip / DNA detection / Lab-on-a-chip
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1 Introduction

The separation and analysis of small DNA fragments have gained much importance in post-human genome project era. 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. This process is mandatory for genomic analysis, DNA fingerprinting, drug discovery, pathogen detection by PCR amplification and environmental analysis and requires conventional gel electrophoresis technique for separation and detection of DNA fragments. Alternatively, such electrophoretic technique can be simplified with the use of narrow capillary channel while applying high electric field across the capillary. A wide variety of charged analytes such as DNA can be separated on the basis of charge-to-mass ratio in these capillaries [1] and are usually detected using optical and or electrochemical method at the end of capillary column [2]. Such CE technique was first introduced in 1960s and since then it has established itself as a powerful analytical tool and has been successfully used in separation and analysis of protein, DNA, sugar, amino acids and pharmaceuticals amongst others [1–3]. Recent advances in microfabrication technique have facilitated the creation of on-chip CE devices coupled with optical and electrochemical detection methods [4–7].

Particularly, the CE analysis can achieve unparalleled sensitivity of up to sub attomoles level when combined with amperometric detection (AD) technique [3]. The amperometric method of detection also offers better chances of miniaturizing CE system compared with optical or spectrometric techniques, owing to the simplicity and ease in microfabrication of on-chip microelectrodes. On the other hand, an optical detection system is difficult to integrate on a microchip and the various sub-components that are required for an optical system add up to the cost of analysis on the fabricated device. Therefore, microfabricated CE devices coupled with amperometric method offer portable low-cost alternative to conventional benchtop-scale analytical equipment [4, 8–10]. However, most of these reported devices suffer the drawbacks of low separation efficiency for closely related analytes and often have low detection sensitivity and non-reproducibility in small microchannel configuration. Alternate methods were devised by various groups with some degree of success by fabricating spiral microchannel configuration in order to pack longer separation length on microdevices [8, 11]. For the analysis of DNA fragments in particular, Joo et al. reported the use of

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Abbreviations: AD, amperometric detection; **CV,** cyclic voltammetry; **PR,** photoresist

Colour Online: See the articles online to view Figs. 1 and 2 in colour.

polyacrylamide gel to increase the separation efficiency [4]. However, this approach required considerably longer analysis time and the method suffered the drawbacks often related to conventional gel-electrophoresis technique. Amongst other approaches, electro-osmotic flow has been used for the separation of DNA fragments in a coated or uncoated capillary, though not in a microchip configuration [12]. Such approach often faces the drawbacks in terms of slow migration of analytes and requirement of very high electric field for separation. Despite the advantages, which an amperometric measurement can offer, the reported systems often relied on end-column optical detection. Moreover, only a few report account for physical explanation pertaining to migratory behavior of analyte inside the microchannel. Such correlation can greatly enhance the sensitivity of detection by accurately calculating the migration time of analyte.

Therefore, in the present work, we devised a simple on-chip CE-AD process for the separation and analysis of DNA fragments in the absence of any polymeric sieving material and successfully correlated its migration process with the existing theoretical models. This approach was helpful in enhancing the detection accuracy in the CE-AD process. The detection of DNA on these CE-AD devices was based on direct amperometric estimation of guanosine and adenosine bases in alkaline condition [13, 14]. Adenosine and guanosine are purine nucleosides and are the building blocks of DNA molecules. These molecules have been previously reported to be electrochemically active [15–17]. Therefore, amperometric analysis of these molecules could provide concentration-dependent correlation on the presence of DNA molecules in CE separation process. For this purpose, a three-electrode system was adopted with Au/Ti in-channel electrodes for direct AD of DNA fragments. The disposable device was fabricated on a glass chip. The capillary microchannels used in this study were engraved in PDMS mold by negative molding method [4]. This method has gained importance due to the suitability of PDMS as an inert and flexible material, which can be fabricated easily, while retaining different shapes in the molded form permanently.

The DNA fragments were separated in these microchannels under low electric field using microfabricated on-chip gold separation electrodes. The electrical mobility of DNA fragments in the capillary has been shown to increase with decreasing molecular weight of analyte [18] and was thus calculated using the following equation:

$$\mu = \frac{L_d}{Et} \quad (1)$$

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) and t is the time required for the sample to travel to the detector (in seconds).

The CE-AD device was subsequently used for the demonstration of its application by detection of samples such as commercial DNA ladder and DNA amplicon from conventional PCR.

2 Experiment details

2.1 Chemicals and apparatus

The testing analytes included guanosine, adenosine, DNA ladder (*Bst*E II digest of lambda phage genomic DNA) and lambda phage genomic DNA from Sigma Aldrich (USA). Custom primers and poly A/T were purchased from Bionics (Korea). Double deionized water was used throughout this study. The positive photoresist (PR) AZ-1512, AZ developer AZ-300 and negative PR used in microfabrication were from Micro-Chem. The negative PR SU-8 2075 and SU-8 developer were procured from the same company. For fabrication of microchannel, the PDMS with brand name Sylgard 184 was procured from Dow Corning (Midland, MI, USA). Photolithography unit was procured from SUSS Microtec. The electrochemical detector having cyclic voltammetry (CV) and *I–t* curve facility was from CH Instruments (USA). The UV ozone cleaner was from Ahtech LTS (Korea).

2.2 CE-AD microchip configuration and fabrication procedure

The configuration of the CE-AD microchip is shown in Fig. 1. The CE-AD microchip was fabricated by standard photolithographic procedures. The chip was built on single sodalime glass substrate using negative molding method involving PDMS polymer for casting the microchannel [4]. For negative molding, a silicon wafer was first cleaned and oxidized with piranha solution (6:1 H_2SO_4/H_2O_2). The wafer was then coated with SU-8-negative PR using a spin coater and patterned using a photomask and UV exposure. The wafer was then hard baked for 30 min at 110°C. The height of the positive patterns on the molding master was 200 µm when measured with a surface profiler. The microchannels were subsequently casted by pouring of degassed mixture of Sylgard 184 silicone elastomer and curing agent (10:1) PDMS on this master wafer, followed by

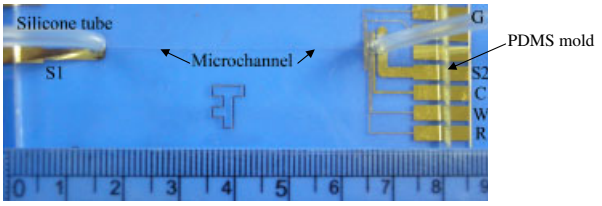

Figure 1. Image of CE-AD microchip showing microchannel engraved in PDMS mold, sample reservoirs, silicon tubes carrying sample and NaOH solution into the microchannel, gold microelectrodes (W, working; C, counter; R, reference; G, decoupler ground electrodes; S1 and S2, separation electrodes) and contact pads.

curing for at least 1 h at 75°C. The positive patterns formed the inverted relief of the desired microstructures and were equal to the channel depth created on the PDMS layer. The cured PDMS was peeled off from the mold, and reservoirs were made at the end of each channel using a 3-mm circular punch. The straight microchannel had the dimensions of 5 cm in length, 250 μm in width and 200 μm in channel height (Fig. 1). The overall dimension of the microchips was 2.5 cm × 6.0 cm with the separation channel located at the center of the chip.

All the electrodes used in this study were made from Au–Ti layers. Three electrodes for the electrochemical detection, namely working, reference and counter; additional two electrodes for applying separation electric field and two decoupler electrodes (Fig. 1) were fabricated on sodalime glass wafer using evaporation method. The decoupling-ground electrodes were positioned in front of the three Au detector electrodes to avoid the interference of high separation electric field on AD (Fig. 1) [4, 8, 19, 20]. The Au layer was fabricated by first spin coating 1.8 μm thick PR (AZ-1512) on the bare glass and then patterning while using a photomask. Subsequently, 50 nm thick Ti and then 320 nm thick Au layers were deposited on the patterned surface. The patterned positive PR was subsequently removed by using ultrasonic cleaner.

The width of reference, working and decoupler electrodes created in this process was 250 μm, whereas for counter electrode was 500 μm. The electrodes were separated by a distance of 200 μm. Separation electrodes had a width of 1 mm each. In the end, the PDMS mold carrying microchannel was bonded with glass substrate containing Au microelectrodes by UV-ozone treatment for 40 min to form the microchip device [4, 21].

2.3 Electrophoresis procedure and electrochemical detection

At first, conventional CV studies were carried out on free guanosine and adenosine bases with a three-electrode system and a CH Instruments electrochemical analyzer (CHI 800B from CH Instruments). The electrochemical experiments were performed in 100 mM NaOH solution with different adenosine and guanosine concentrations at various scan rates using conventional (gold working, Pt counter and Ag/AgCl reference) electrodes from CH Instruments. Through this CV experiments, we could find the detection voltage(s) to be applied in CE-AD device and peak current range that these chemicals would generate. For further confirmation of peak detection voltages, a 5-mL NaOH solution containing 5 μg of commercial fish sperm DNA (Sigma Aldrich) was subjected to similar cyclic voltammetric analysis.

Subsequently, the disposable microchip was subjected to CE-AD separation analysis of free bases and samples of DNA while applying the detection voltages as obtained from their cyclic voltammograms. At first, NaOH solution, which was the separation medium and the supporting electrolyte, was filled in the microchannel using silicone tubes and precision syringe pump (KD Scientific, USA) while avoiding air bubble formation inside the channel. The volume of each reservoir was about 35 μL. These were completely filled with NaOH solution to avoid negative hydrodynamic pressure on electrophoretic migration of analytes due to volume difference between reservoirs. Then 1 μL of the sample was injected into sample reservoir (filled with NaOH) close to the microchannel opening using a micropipette and an electric field of 100 V was applied immediately between the inlet reservoir and the waste reservoir. Which means the sample was diluted 35 times in the reservoir, albeit as a result of slow diffusion. Since separation potential was switched on immediately after sample addition, thereby limiting the rate of sample mixing into reservoir; therefore, the concentration of the analyte that we reported throughout this study represented 1 μL volume that was injected into the reservoir. In addition, the liquid in the reservoirs did not dry up due to evaporation even until 6 h of operation, thereby allowing sufficient time to conduct CE-AD process. AD was performed with three-electrode configuration (Fig. 1) placed in the path of analyte flow. Redox reaction of adenosine or guanosine from testing analytes on the working electrode generated current peaks in the amperometric *I–t* curve, which was recorded and stored on a computer using the electrochemical analyzer.

2.4 Analysis of DNA samples

The CE-AD microchip was used in the analysis of single- as well as double-stranded custom DNA, commercial DNA ladder as well as amplified DNA sample from PCR. For this purpose, first, 1 μL of a mixture containing 25-mer polyA single-stranded DNA (ssDNA), 25-mer, 50-mer and 100-mer polyAT double-stranded DNA (dsDNA) of 500 pmol concentration each was subjected to CE analysis using the microchip. The separation time for this sample was recorded along with the peak detection current in the form of an electropherogram. For exemplifying the usefulness of the proposed method, the device was tested for separation and detection of commercial DNA ladder and latter for PCR DNA sample. For the detection of DNA ladder, 1 μL sample (1 μg/μL) of *Bst*E II (a restriction enzyme for cutting DNA into fragments of known sizes) digest of lambda phage genomic DNA was subjected to CE-AD analysis. For analyzing PCR samples, the genomic DNA of virus λ-phage was subjected to PCR reaction in a conventional thermocycler for 30 cycles using forward and reverse primer, 5′-GCA-AGT-ATC-GTT-TCC-ACC-GT-3′, and reverse primer, 5′-TTA-TAA-GTC-TAA-TGA-AGA-CAA-ATC-CC-3′ as per previously reported procedure [22]. The PCR amplicon (1 μL) was analyzed on CE-AD microchip against pre-PCR mix as the control. The amplicons were then also analyzed through conventional agarose gel electrophoresis [23] and confirmed by gel-doc imaging.

3 Results and discussion

3.1 Device fabrication

The CE-AD microchip used in this study was fabricated on a glass substrate with PDMS mold (5 mm thick) containing microchannel pattern. The separation microchannel was produced by negative molding process [4] using PDMS mold formed onto a microfabricated molding master. The PDMS is the widely used material for microfluidic devices especially due to their transparent and flexible properties and as they can retain microstructures for a considerably long period without fatigue. The PDMS is also an inert material and has been shown as not interfering with CE-AD procedure [8].

The choice for all-gold electrode material was due to their inertness to redox reactions and ease in fabrication over having conventional Au, Pt and Ag/AgCl electrode configuration. Gold is also an excellent material for fabricating working as well as pseudo-reference electrode through simple photolithographic techniques resulting in readily produced micrometer-sized conducting metal film electrodes [19, 20]. It can be directly laid on glass substrate via thermal evaporation.

The design of our CE-AD separation channel was considerably different from previous reports incorporating double-T structure where samples are added in a reservoir perpendicular to the main separation channel [24]. In such configuration, the sample is first pre-concentrated in the main channel before being subjected to electrophoretic separation. While a double-T injector configuration is more commonly reported throughout the literature, this configuration requires prior knowledge of migration time for analytes to some extent. In an event where more than a few unknown species are present in sample, for instance, DNA ladder containing DNA fragments of different lengths, double-T configuration is not easy to implement. Therefore, in this study, we adopted a simple straight channel configuration for ease in operation and effective separation of complex samples such as DNA ladder.

3.2 CV of adenosine and guanosine

The cyclic voltammetric analysis of adenosine and guanosine bases was the pre-requisite for the knowledge of detection voltage to be applied in CE-AD procedure and peak current range that these chemicals would generate. In addition, if a nucleic acid such as DNA fragment has to be detected, the cumulative amperometric peak would provide detailed information on the length of nucleotide and the concentration of either of the purine bases. Therefore, conventional CV analysis of adenosine and guanosine was performed using three electrodes (gold working, Pt counter and Ag/AgCl reference) in a beaker. The NaOH was used as the medium as well as supporting electrolyte because adenosine and guanosine molecules have poor solubility in water. Other supporting electrolyte such as KCl was avoided due to similar solubility problem. The NaOH solution also proved useful, as nucleosides and DNA molecules were redox inactive at neutral or acidic pH, under present experimental conditions. This was in slight contradiction to previous claims regarding redox properties of DNA at neutral pH [17, 25]. However, the electrochemical behavior of nucleosides as well as DNA molecules was excellent in NaOH solution. Moreover, DNA is relatively resistant to alkaline hydrolysis and related denaturation [26], hence further experiments were carried out in this medium only.

The electrochemical experiments were performed in 100 mM NaOH solution with different concentrations of nucleosides sample (Fig. 2). The electrochemical measurements were performed at least three times for each condition ($n = 3$) except otherwise stated. The potential was cycled from +1.5 to 0.5 V with a scan rate 100 mV/s for adenosine. In case of guanosine CV, the condition was +1.0 to −0.4 V with a scan rate 100 mV/s. Figure 2A and B shows the cyclic voltammograms of adenosine and guanosine on the gold electrode in 100 mM NaOH solution (anodic current is shown in negative

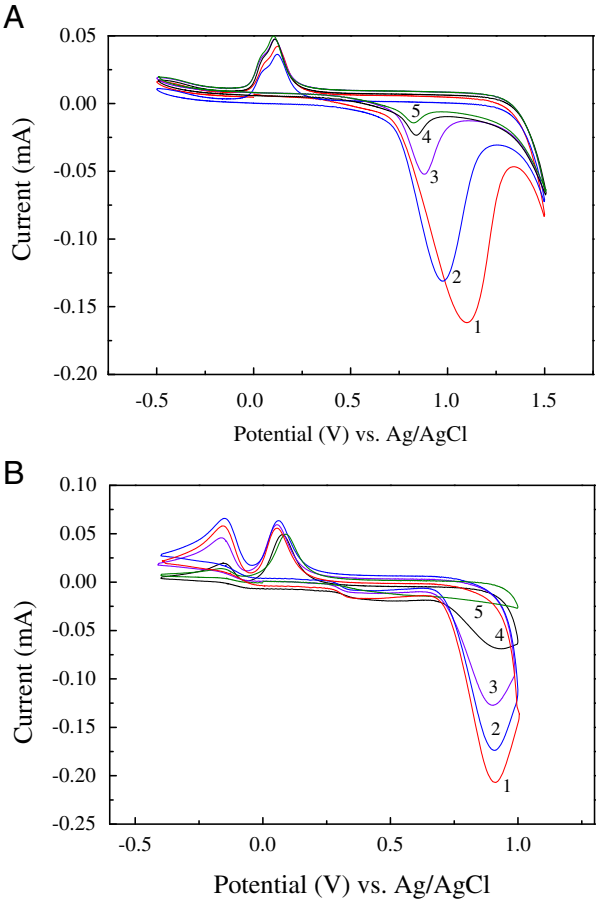

Figure 2. CV of nucleosides at different concentrations (1: 1 mM, 2: 100 μM, 3: 10 μM, 4: 1 μM and 5: 100 nM) using conventional disc electrodes of 2 mm diameter and reaction vessel from CH Instruments: (A) adenosine and (B) guanosine in 100 mM NaOH; scan rate: 100 mV/s.

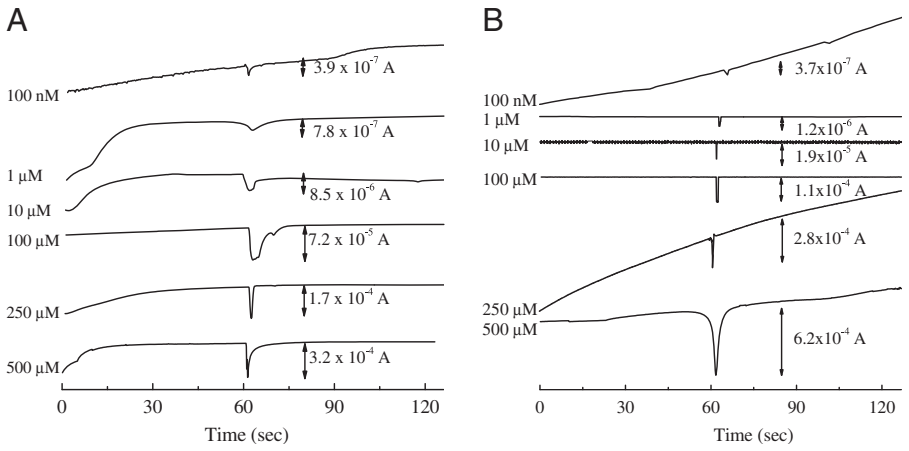

Figure 3. Electropherograms of (A) adenosine and (B) guanosine at 100 nM, 1 μM, 10 μM, 100 μM, 250 μM and 500 μM concentrations at separation voltage: 100 V dc, detection voltage: 0.92 and 0.82 V (respectively).

y-axis). Adenosine and guanosine concentration used was 1 mM, 100 μM, 10 μM, 1 μM and 100 nM. Both adenosine and guanosine produced defined oxidation peaks in the anodic scan at 0.97 and 0.82 V, respectively, with peak currents directly proportional to its concentration. These oxidation peak voltages were subsequently applied for CE-AD analysis without correction for negative hydrodynamic pressure, as our microchip configuration did not suffer from this drawback. On the other hand, the cyclic voltammogram of fish sperm DNA carried out under similar conditions also showed peak detection potentials corresponding to adenosine and guanosine (see Supporting Information). This further strengthened the possibility to use these peak detection voltages in CE-AD analysis of DNA.

3.3 CE-AD of adenosine and guanosine

Unlike previous studies, involving CE-AD detection of DNA fragments, our device microchannels needed no preparation steps or preconditioning prior to use. Also, there was no necessity for use of polymeric gel such as polyacrylamide to achieve desired level of separation efficiency [4]. The CE-AD separation analysis was carried out by injecting separation medium NaOH into the microchannel before loading the sample. Adenosine and guanosine were subsequently analyzed on the microfluidic chip at their peak detection voltages. The separation voltage was kept at 100 V, thus, effectively creating field strength of 20 V/cm, which is one of the mildest conditions used in any CE-AD analysis. A low separation voltage ensures less shearing forces experienced by biomolecules, thereby preserving their native state. The resulting electropherograms are shown in Fig. 3A and B. In these experiments, the peak and the migration time of adenosine (60.8 s, SD = 3.2 s, $n = 3$) and guanosine (62.8 s, SD = 2.9 s, $n = 3$) can be seen nearly similar, as the molecular weights of these nucleosides are very close to each other. These electropherograms also indicate the efficiency of using small concentration and volume (1 μL) in a CE-AD device due to microampere level detection. The increasing concentration of purine nucleosides also

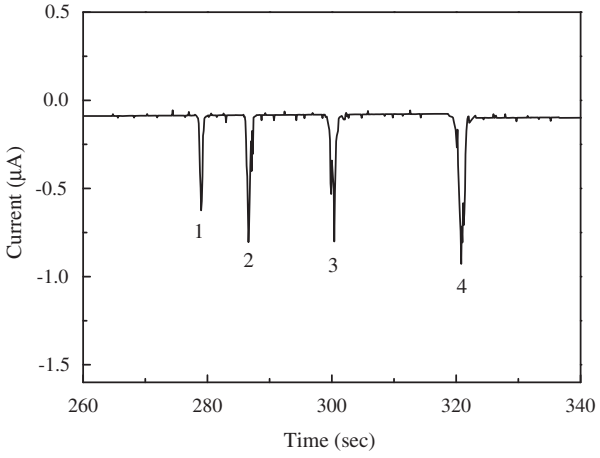

Figure 4. Electropherogram of a mixture containing 500 pmol each of (1) 25-mer polyA ssDNA; (2) 25, (3) 50 and (4) 100-mer poly AT dsDNA at separation voltage 100 V dc and detection voltage 0.92 V.

increased the detection current level in a linear manner with a linear range of 0.1–500 μM (data not shown). The slopes and the intercepts for linear fit (using the software Origin™ version 8) of their correlation were 6.61×10^{-7} μA/μM and 1.06×10^{-6} μA (regression coefficient 0.998) for adenosine and 1.15×10^{-6} μA/μM and -1.81×10^{-7} μA (regression coefficient 0.995) for guanosine, respectively. These results suggested the feasibility of CE-AD device in the analysis of ssDNA as well as dsDNA samples.

3.4 CE-AD analysis of single- and double-stranded DNA samples

The aim of the present study was to separate and analyze nucleic acid fragments by CE-AD method. The success of this methodology would decide the usefulness of the proposed device in rapid detection of pathogen, probe hybridization and related molecular biology techniques. Therefore, we first attempted to analyze a 1-μL mixture containing 500 pmol each of 25-mer ss-polyA and 25- to

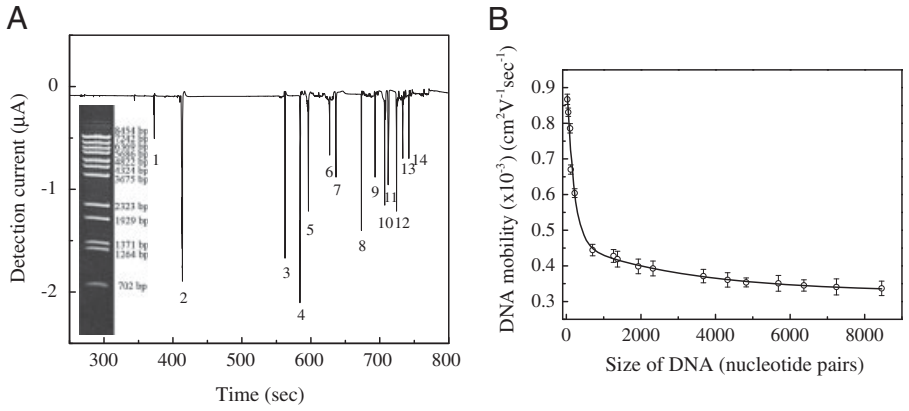

Figure 5. (A) Electropherogram of CE-AD separation of commercial DNA ladder (migration time for 14 fragments shown as numbers) at separation voltage of 100 V dc and detection voltage of 0.82 V (inset shows gel doc of DNA ladder on a 7% agarose gel); (B) DNA mobility coefficient for each DNA fragment calculated from Eq. (1). The converged curve fit used exponential decay-3 equation model from Origin^{TM} software (version 8).

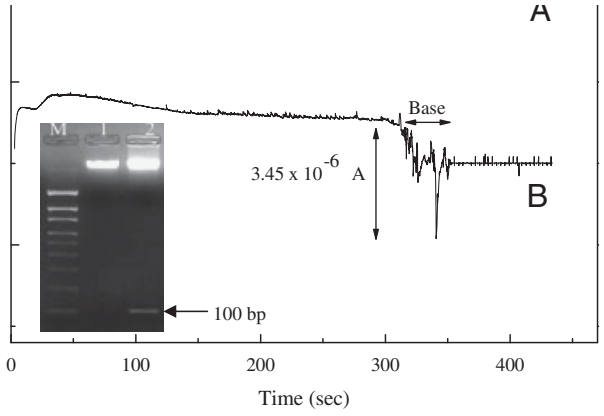

Figure 6. PCR amplification of λ-phage genomic DNA using custom primers and conventional thermocycler. CE-AD analysis of (A) pre-PCR mix (not subjected to PCR) and (B) product from 30 cycles of PCR amplification at separation voltage 100 V dc and detection voltage 0.82 V (for guanosine); (inset) gel-doc of agarose gel electrophoresis for the confirmation of PCR reaction (6 μL sample loading): Lane M = 1 kb DNA marker, lane 1 = pre PCR mix and lane 2 = product from 30 cycles of PCR.

100-mer ds-polyAT. The electrophoretic separation of these molecules at 100 V DC separation voltage and 0.92 V detection voltages (for estimating adenosine) yielded electropherograms as can be seen from Fig. 4. The DNA fragments had different net charge and molecular weights; hence, their electrophoretic separation peaks appeared at different migration times. This proved the feasibility of the proposed device for the separation and analysis of DNA of various lengths. Therefore, we next analyzed a commercial DNA ladder containing a mixture of 14 different fragments of DNA. The CE-AD analysis could successfully separate and analyze the DNA fragments (Fig. 5A). The migration coefficient (DNA mobility) for these fragments could be calculated from Eq. (1), which fully satisfied the existing theoretical models for such capillary electrophoretic separation [18] albeit in an exponential manner (Fig. 5B). Such comparison between migration time and DNA mobility is rarely used in the reports citing similar work, though such a calculation is necessary to enhance the reliability and detection sensitivity of CE-AD device, especially for the sake of deducing DNA length from their migration time.

The CE-AD device was further tested for the detection of realistic sample of DNA. PCR is amongst one of the reactions used extensively in molecular biology, forensic and clinical analysis protocols. In this reaction, known stretches of DNA are enzymatically amplified to several folds in the form of open-ended short dsDNA fragment and the presence of amplified DNA or amplicons is verified using agarose gel electrophoresis. In order to exemplify an alternative to this long and tedious procedure, we amplified a short stretch of λ-phage viral DNA by a conventional PCR machine and analyzed the amplicon on our CE-AD microchip along with a suitable control (pre-PCR mix not subjected to PCR reaction). The intended amplicon size was 100 bp dsDNA. The electropherogram thus obtained confirmed successful detection of 100 bp PCR product (Fig. 6). The length of DNA as calculated from Fig. 5B for its migration time of 329.5 s (mean from its peak base) was 104 bp. This proved the successful miniaturization of a CE process as a microchip, which was also an effective DNA analysis platform, and this opened the possibility for further applications of proposed microchip.

4 Concluding remarks

In conclusion, we have demonstrated in the present work, a method for the analysis of ssDNA and dsDNA using CE with AD system. The device could successfully detect purine nucleosides as well as ssDNA and dsDNA after their electrophoretic separation in the microchannel. Unlike previous studies, the device did not require any preconditioning or use of polyacrylamide gel in the microchannel. The success of this work provides great opportunity to

separate various DNA fragments and biological molecules on a microchip. Therefore, we believe that the CE-AD microchip can be a viable candidate for the fabrication of portable lab-on-a-chips involving rapid detection of DNA in a variety of samples.

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The authors have declared no conflict of interest.

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