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Detection of bacterial pathogen DNA using an integrated complementary metal oxide semiconductor microchip system with capillary array electrophoresis

Joon Myong Song, Joel Mobley, Tuan Vo-Dinh*

Advanced Biomedical Science and Technology Group, Life Sciences Division, Oak Ridge National Laboratory, PO Box 2008, Oak Ridge, TN 37831-6101, USA

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Abstract

In this paper, we show an integrated complementary metal oxide semiconductor (CMOS)-based microchip system with capillary array electrophoresis (CAE) for the detection of bacterial pathogen amplified by polymerase chain reaction (PCR). In order to demonstrate the efficacy of PCR reaction for the heat-labile toxin producing enterotoxigenic *Escherichia coli (E. coli),* which causes cholera-like diarrhea, 100 bp DNA ladders were injected along with the PCR product. Poly-(vinylpyrrolidone) (PVP) was used as the separation medium and provided separation resolution which was adequate for the identification of PCR product. The miniaturized integrated CMOS microchip system with CAE has excellent advantages over conventional instrumental systems for analysis of bacterial pathogens such as compactness, low cost, high speed, and multiplex capability. Furthermore, the miniaturized integrated CMOS microchip system should be compatible with a variety of microfabricated devices that aim at more rapid and high-throughput analysis.

Keywords: Complementary metal oxide semiconductor microchip; Polymerase chain reaction; DNA; Poly(vinylpyrrolidone)

1. Introduction

Over the past decade, capillary array electrophoresis (CAE) has been actively used as a powerful tool to overcome limitations of the slab gel electrophoresis, which has proven to be insufficient in meeting the demands for rapid and high-throughput analysis in modern biology. High electric fields and a large number of capillaries in CAE increase the speed and the throughput of electrophoretic separations, respectively. A variety of biomedical applications of CAE to DNA sequencing [1–5], genotyping [6,7] and mutation analysis [8] have been reported.

The microfabricated devices have obviously contributed to the significant improvements in rapid and high-throughput analysis in modern biology [9–16]. The miniaturization efforts of microfabricated devices have mainly been associated with analysis systems including the electrophoretic separations or

*Corresponding author: Tel.: +1-865-574-6249; fax: +1-865-576-7651.

E-mail address: vodinht@ornl.gov (T. Vo-Dinh).

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supported specific immobilization reactions. Until now, the detectors and associated optical systems have received little focus in development of miniaturized systems using microfabrication techniques. Accordingly, there is still room for further development of a more miniaturized system. In conventional microfabricated devices, photomultiplier tubes (PMTs) and charge-coupled devices (CCDs) have been used as the main detectors for laser-induced fluorescence (LIF), these permit highly sensitive detection of biological samples such as DNA and protein. The PMT that performs single-channel detection can be combined with the confocal scanning technique for the high throughput LIF-based detection. Although the confocal scanning technique provides highly sensitive detection, microchip translation is required [17]. Compared to a fixed optical geometry, this could be a disadvantage in the development of a fully miniaturized system. Due to its high throughput and sensitivity, CCDs have been actively used for the rapid and high-throughput analysis. However, CCDs are large when compared to microchips. Furthermore, they are expensive and complex to use. Accordingly, there is demand for the development of a miniature, cost-effective detection system for use with microchip platforms.

Recently, our group has developed an integrated circuit (IC) microchip sensor using the complementary metal oxide semiconductor (CMOS) technology [18–21]. This CMOS technology allows for our microchip system to be fabricated inexpensively and allows for low power consumption during operation. Most optical detection systems are relatively large when the amplifier units and power supply for detectors are included, which makes them practical only for laboratory usage. However, in our microchip system, a 4×4 array of photodiode detection elements, amplifiers, discriminators, and logic circuits are included on board in a single IC package. This integration provides a miniaturized detection device, which can contribute greatly to the detection system of a fully miniaturized system. Previously, this inexpensive and miniaturized system was successfully applied to the analysis of target genes based on DNA probe microarrays [18–21]. In this work, another useful development of the CMOS microchip system is demonstrated by combining it with CAE to provide a mobility-based separation for a wide range of biomedical applications.

Microorganisms such as bacteria exist widely throughout nature and the environment. The many microorganisms perform essential activities in nature and provide plants and animals with beneficial relations. However, a lot of harmful microorganisms can have serious effects on animals and human beings, often resulting in infectious diseases. The bacteria can spread easily and quickly through water and food with the appropriate temperature and moisture conditions. The harmfulness of pathogenic bacteria is demonstrated by the fact that infectious diseases are involved in nearly 40% of the total 50 million annual estimated deaths [22]. A variety of methods such as IR identification [23], flow cytometry [24] and biosensors [25,26], have been developed for rapid, sensitive, and selective bacterial detection.

In this paper, we present the detection of polymerase chain reaction (PCR)-amplified bacterial pathogen DNA using the integrated CMOS microchip system with CAE. Currently, PCR is one of the most important tools in every aspect of biology that depends on genetic materials. The great advantage of PCR is in the rapid and specific amplifications of target genes through a cyclic and enzyme-catalyzed reaction. PCR techniques have been actively applied to forensic DNA typing, mutation analysis, and clinical diagnosis. In this work, it is shown that the integrated biochip system with CAE can be successfully applied to biomedical research through the detection of PCR-amplified bacterial pathogen DNA with high speed and multiplex capability. Also, the use of the miniaturized CMOS microchip system as a detector in CAE shows a great potential for compatibility with conventional microfabricated devices, which should contribute to further development of a miniaturized system for more rapid and high-throughput bioassays.

2. Experimental

2.1. CMOS microchip instrument

The microchip in this work was fabricated using the CMOS technology in our laboratory. The CMOS

microchip is a single integrated circuit (IC) package that contains a 4×4 array of photodiodes and photodiode element addressing circuitry with signal processing. The advantages of the CMOS-based system include its operation using low supply voltages and low cost fabrication. The dimension of each photodiode is 900×900 μm. The distance between two photodiodes is 100 μm. A National Instruments DAQ516 PCMCIA card installed in a laptop computer provided digital I/O lines and an analog-to-digital conversion channel so that the CMOS microchip detection elements were individually accessed and read out. The gain of the CMOS microchip from unit to 100 was controlled using a logic circuit in the microchip system. A custom written software interface constructed with LabView controlled the data acquisition process for the CMOS microchip system.

2.2. PCR reagent mixture and protocol

The target template, enzyme, buffer, dNTP mixture, and a set of primers for the PCR bacterial pathogen detection were purchased from Panvera (Madison, WI). The target template was a gene sequence of enterotoxigenic *Escherichia coli (E. coli)* that produces the heat-labile enterotoxin. The PCR reagent mixture was composed of (a) 1 μl of positive control DNA EC1 (storage buffer: 10 m*M* Tris–HCl, 1 m*M* ethylenediaminetetra-acetic acid (EDTA), pH 8.0); (b) 10 μl of 10×PCR buffer (20 m*M* Mg^{2+}); (c) 8 μl of dNTP mixture (2.5 m*M* of each dNTP, solved in water, pH 7~8); (d) 1 μl of primer ELT-1 and ELT-2 (19 pmol/μl, in sterilized water); (e) 0.5 μl of TakaRa Ex Taq™ (5 units/μl, storage buffer: 20 m*M* Tris–HCl, 100 m*M* KCl, 0.1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 0.5% Tween 20, 0.5% Nonidet P-40, 50% glycerol, pH 8.0); (f) 78.5 μl of sterilized water. The above solutions were stored on ice during the preparation of the PCR mixture. The amount of target template in the PCR solution was 100 pg. The total volume of PCR solution was 100 μl. The PCR machine (TempCycler II) was purchased from COY Corporation (Grass Lake, MI). The PCR was performed with the following protocol: 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min.

2.3. Chemical reagents and buffer

Rhodamine 610 was obtained from Exciton (Dayton, OH). 100 bp DNA ladders (500 ng/μl) were purchased from New England Biolab (Beverly, MA). Further dilution of DNA ladder solution was performed with 1×TBE buffer. 1×TBE buffer was prepared by dissolving 16.86 g premixed TBE powder (Amerosco, Solon, OH) in 1000 ml of deionized water (pH ~8.3). 1×TBE buffer consists of 89 m*M* Tris(hydroxymethyl)aminomethane (THAM), 89 m*M* boric acid, and 2 m*M* EDTA. Methanol was obtained from Fisher (Fairlawn, NJ). Poly(vinylpyrrolidone) (PVP) (M_r, 130 000) was purchased from Aldrich (Milwaukee, MI) and ethidium bromide was obtained from Molecular Probes (Eugene, OR). Powder of ethidium bromide (0.5 mg) was dissolved in 1000 ml of 1×TBE buffer so that the final concentration was 0.5 μg/ml. This solution was used as a running buffer. The sieving matrix was made by dissolving 4.5% PVP in 1×TBE buffer with ethidium bromide of 0.5 μg/ml by soft shaking for 15 min. The capillaries (75 μm I.D., 365 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ). The effective length of separation capillaries was 55 cm.

2.4. Capillary array electrophoresis

The detection windows of four capillaries were made by burning off the polyimide coating and were washed with methanol-soaked lens cleaning paper. Four capillaries were then closely packed side by side at the detection windows. A capillary holder that holds the packed capillaries was mounted on a translational stage and positioned so that the capillary array was parallel to the optical bench. Four capillaries were filled with the sieving matrix using a 100 μl syringe (Hamilton, Reno, NV) and the ends of capillaries were inserted into buffer reservoirs. The electrodes were connected to a DC power supply (Hipotronics, model: R30B, Brewster, NY). Before sample injection, separation electric field was applied to the capillaries for 20 min by the power supply and then electrokinetic injection of DNA samples into the capillaries was performed at 10 kV for 5 s. For the capillary column regeneration, the sieving matrix was flushed out with water using a 100 μl syringe.

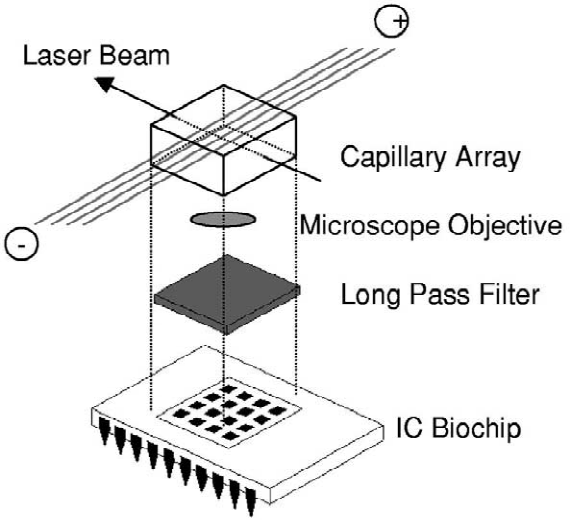

Fig. 1. A schematic diagram of the experimental apparatus.

2.5. Laser-induced fluorescence

Fig. 1 presents a schematic view of the integrated CMOS microchip system with CAE. A 514.5 nm beam from an Argon-ion laser (10 mW; Omnichrome 532, Chino, CA) was separated from its plasma emission by a 60° equilateral prism (Edmund Industrial Optics, Barrington, NJ). An iris diaphragm was used to eliminate extraneous light from the laser. The laser beam was focused onto the capillary array using a lens with 40 mm focal length in order to irradiate all the detection windows of the capillary array. A capillary holder was adjusted using a translational stage so that the laser beam passed through the center of the capillaries. Fluorescence from the capillary array was collected with a ×5 microscope objective (Nikon, 0.1 NA, Melville, NY) and detected with the CMOS microchip that was perpendicular to the multiplex capillary. A long pass optical filter (cut-off position: 590 nm, Edmund Industrial Optics) was attached on the CMOS microchip to eliminate the laser scattering.

3. Results and discussion

In the present development, it is important to achieve a compact optical geometry for the construction of a miniaturized system. The dimensions of photodiodes in the CMOS microchip and the individual capillaries of the capillary array are important factors to consider for successful design of the compact optical geometry. Compared to the conventional CCD, the CMOS microchip has much larger pixel dimensions (900×900 μm) than a typical CCD (~ 20×20 μm). In the conventional CAE with CCD detection, the dimension of a single capillary (365 μm O.D., 75 μm I.D.) is much larger than that of one pixel of CCD. Accordingly, the ideal optical coupling between capillary array and CCD is achieved when the smallest possible number of pixels corresponds to the image of a single capillary for high throughput. However, because the pixel dimension of CMOS microchip is larger than the outer diameter of single capillary, a 1-to-1 correspondence between CMOS microchip pixels and capillaries can be achieved, even with magnification of the capillary. To satisfy this condition, use of the microscope objective was the most ideal solution that fulfilled above requirements due to its compactness and magnification capability. Based on the dimensions of the capillaries and CMOS microchip pixels, a ×5 microscope objective was selected for the optical coupling between the capillary array and the photodiode array as shown in Fig. 2. With this optical geometry and the large distance (100 μm) between two pixels, each photodiode detection element received minimal stray light from adjacent capillaries, which contributed to negligible cross-talk. Another factor to consider for achieving the compact optical geometry was simultaneous focusing of a single laser beam focusing onto the capillary

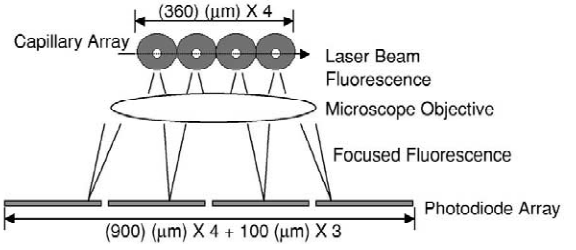

Fig. 2. An optical coupling between the capillary array and the photodiodes in the CMOS microchip. The capillary array images were magnified by a ×5 microscope objective, provided a 1-to-1 correspondence between capillaries and photodiodes.

array. This was accomplished by optimizing the laser beam focus so that each capillary could act as a lens. In this case, the laser beam is focused onto an outermost capillary, and the beam is propagated through the array in nearly collimated fashion. The simultaneous beam focusing was combined with the small dimension of the objective lens and the microchip detector to form a miniaturized system which was ideal for CAE in rapid and high-throughput bioanalysis.

Fig. 3 shows the simultaneous fluorescence detection of Rhodamine 610 obtained with the CMOS microchip system after the capillary array was filled with an aqueous solution of 1×10^{-6} *M* Rhodamine 610. This figure shows the response of four photodiodes detection elements. The laser beam irradiation onto the capillary array was controlled by a mechanical shutter. When the laser beam was irradiating the capillary array, the four photodiodes showed obvious optical response. The dark current signal was obtained when there was no laser beam irradiation onto the capillary array. The CMOS microchip, held by a translational stage, was moved in three dimensions to find the optimized focal plane and alignment of the four fluorescence images from the four capillaries. The fluorescence intensities of Rhodamine 610 decreased with the number of capillaries that the laser beam passed through. The DNA analysis was performed after this optical adjustment for the detection of the highest fluorescence signal.

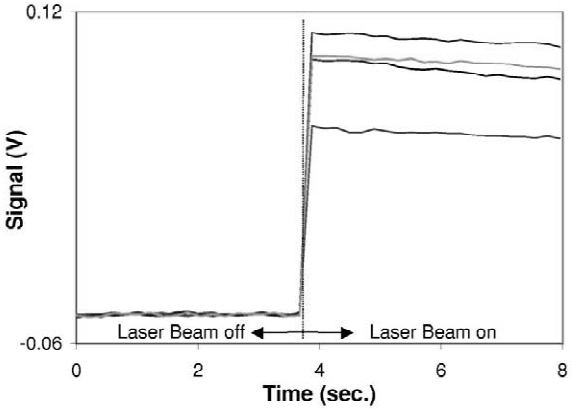

Fig. 3. Optical response of photodiode array in the CMOS microchip. Four of the 16 photodiodes were used to record the fluorescence signals of Rhodamine 610 from the capillary array after the optical adjustment. When the laser beam did not irradiate the capillary array, the four photodiodes showed dark current signals.

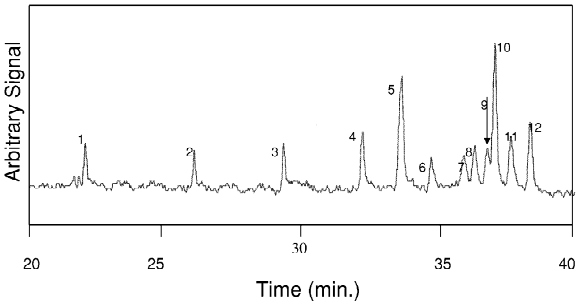

Fig. 4. Separations of 100 bp DNA ladders in a single capillary using 4.5% PVP and 180 V/cm electric field. The CMOS microchip was operated with 100 gain and 5 Hz data acquisition rate. The DNA sample concentration was 25 ng/μl. Peak legend: (1) 100, (2) 200, (3) 300, (4) 400, (5) 500, (6) 600, (7) 700, (8) 800, (9) 900, (10) 1000, (11) 1200, (12) 1517 bp.

Fig. 4 shows the separations of 100 bp DNA ladders in a single capillary obtained with the CMOS microchip system with gain set to 100. 4.5% PVP was used as the separation medium and an electric field of 180 V/cm was applied for DNA separations. The PVP was selected as the separation medium based on its very low viscosity and excellent dynamic coating property on the capillary inner wall. This property is very suitable for the capillary column regeneration [27]. The most important factor in DNA separation is in the mesh size of polymeric gel with appropriate sieving effect on the DNA fragments. Except for 900 and 1000 bp DNA fragments, all the DNA ladders from 100 bp to 1517 bp were baseline resolved. This condition was used for the analysis of PCR-amplified bacterial pathogen DNA.

High speed and sensitivity are the most important factors to be considered in the bacterial detection because even a single pathogenic organism can result in an infectious dose to human body. The selectivity in bacterial detection is also important due to the condition that low numbers of pathogenic bacteria coexist with non-pathogenic organisms in a complex biological environment. Of methods for the bacterial detection, IR identification is based on all the individual molecular components of the microorganism using the IR cell absorption spectra. On the other hand, flow cytometry uses light scattering of

cells stained with dyes, which provides very useful information on cell size, shape, and bacterial growth. Flow cytometry is very suitable for the rapid analysis of individual cells. Bacterial detection using biosensors is generally based on bioreceptors such as probe genes and antibodies that perform biological recognition. Biosensors promise minimum sample treatment and the detection of a broad spectrum of analytes in complex sample matrices. Recently, PCR techniques for the detection of bacterial pathogen have been actively used due to their precision, selectivity, and high sensitivity. These advantages allow extremely specific identification, even allowing discrimination between closely related organisms. The detection of bacterial pathogen DNA after PCR reaction is normally performed via slab gel electrophoresis. However, slab gel electrophoresis is fairly insensitive and requires long separation times, which imposes significant limitations on the overall efficiency of PCR techniques for high speed and sensitive analysis. Another approach for detection of post-PCR bacterial pathogen DNA is to use a nucleic acid microarray. This technique has excellent capability to selectively identify and detect PCR products using DNA sequence differences, but individual probe genes have to be prepared for each PCR product, which is expensive and time-consuming. In terms of rapidity, high-throughput, low cost, and high sensitivity, the integrated CMOS microchip system with CAE can provide an excellent alternative to the slab gel electrophoresis and DNA probe microarray techniques for detection of post-PCR bacterial pathogen DNA. Another advantage of the CMOS microchip-CAE system is that capillary arrays are reusable unlike DNA microarray.

Fig. 5 shows the simultaneous detection of PCR-amplified bacterial pathogen DNA in the capillary array obtained with the integrated CMOS microchip system. As optimized for the separation of DNA fragments in a single capillary described above, 4.5% PVP and 180 V/cm were used to separate DNA fragments in the capillary array. Electropherograms at capillary number 1 and number 2 represent the PCR product of enterotoxigenic *E. coli* that causes cholera-like diarrhea in humans and animals [28–30]. This PCR amplification was performed based on the extracted genomic DNA from enterotoxigenic *E. coli* as template, which is produced by a manufacturer. Enterotoxigenic *E. coli* produces enterotoxins such as heat-labile toxin and heat-stable toxin. The electropherograms of PCR product at the capillary number 1 and number 2 correspond to gene amplified by a set of primers associated with the heat-labile toxin. The size of PCR product is 690 bp according to the specification by manufacturer. The electropherogram at capillary number 3 shows separations of 100 bp DNA ladders in 4.5% PVP sieving matrix and 180 V/cm electric field. The migration

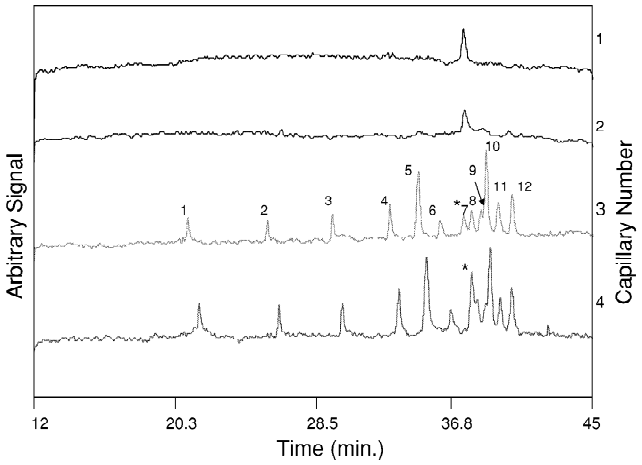

Fig. 5. Simultaneous four-channel detection for the analysis of PCR-amplified *E. coli* gene. The separation conditions applied to four capillaries was 4.5% PVP and 180 V/cm electric field. Peak legend in the electropherogram at capillary number 3: (1) 100, (2) 200, (3) 300, (4) 400, (5) 500, (6) 600, (7) 700, (8) 800, (9) 900, (10) 1000, (11) 1200, (12) 1517 bp.

times of 100 bp DNA ladders can be used for the identification of PCR product based on size. The size of DNA ladder closest to 690 bp PCR product in size is 700 bp, which has 10 bp size difference. However, it is difficult to accurately perform the sizing analysis of the PCR product based on the migration times of DNA ladders and PCR products in capillary number 3 and capillary number 2 respectively, due to the differences among migration times of DNA fragments between different capillaries, induced mainly by the inhomogeneities in gel matrices and wall conditions. Accordingly, the size analysis of PCR products needs to be confirmed further. The co-injection of PCR product and DNA sizing markers is a very useful method for the identification of PCR product. The electropherogram at capillary number 4 corresponds to the co-injection of PCR product of enterotoxigenic *E. coli* gene and 100 bp DNA ladders. The electropherogram shows a spike induced by the 690 bp PCR product around the migration time between 600 bp and 800 bp DNA fragments. This spike shows the efficacy of PCR reaction for the heat-labile toxin-producing, enterotoxigenic *E. coli* gene. This is especially illustrated by the signal ratios between 700 bp and 800 bp DNA fragments in electropherograms at capillary number 3 and number 4. Unlike the electropherogram at capillary number 3, the 800 bp DNA ladder shows smaller signal than the faster peak that corresponds to 700 bp DNA ladder and 690 bp PCR product in the electropherogram at capillary number 4. This integrated CMOS microchip system with CAE has several advantages over conventional biosensors for detection of bacterial pathogens, including high speed, low cost, compactness, and multiplex capability. These features are very suitable for the construction of a fully automated miniaturized system.

4. Conclusion

Due to its compact design and multiplex capability, the CMOS microchip system combined with CAE provided high speed and throughput analysis as a new tool for the detection of bacterial pathogen. Based on its compactness, low cost, and multiplex capability, the integrated CMOS microchip system as a detector is expected to be compatible with conventional microfabricated devices to allow more rapid and high throughput analysis.

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