

Integrated Circuit Bipolar Semiconductor Microchip-based Optical Detection of Biomolecules: Parallel Applications as a Miniaturized Sample Platform and a Two-dimensional Photodetector

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Abstract

Recently, our laboratory developed a photodiode array (PDA) microchip based on bipolar semiconductor technology. A 12×12 micro array of photodiodes, an array of current amplifiers, and a photodiode element-addressing circuit were integrated into a single IC chip. Each photodiode had dimensions of $300 \times 300 \mu\text{m}^2$ and the photodiode-to-photodiode distance was $100 \mu\text{m}$. The chip was successfully applied to the on-chip detection of target DNA and protein based on optical transduction detection method. This report demonstrates the compatibility of the constructed PDA chips to the on-chip reaction conditions and the effects of operational parameters that may lead to false data interpretation. The chip has also been used as a two dimensional photodetector in a compact optical system to detect *Escherichia coli* O157 : H7 based on the combined use of ELISA and laser-induced fluorescence (LIF). This work shows that the PDA microchip system has the potential to be used as a highly compact and inexpensive alternative to CCD detection for spatially separated bio-reactors.

Key words: Bipolar semiconductor microchip, On-chip assay, Enzyme-linked immunosorbent assay (ELISA), Pathogenic bacteria detection

Introduction

In the development of biosensors, optical transduction methods have gained much attention due to their attractive advantages¹⁻⁷. The key component of a biosensor is a bioreceptor, such as antibodies or DNA

strands, which permits selective binding to, and recognition of specific biomolecules.

Bioreceptors are usually attached to a solid transducer surface that is in contact with an analyte. Analyte detection is achieved by measuring the degree of binding of the analyte molecules to the surface-bound bioreceptors. The reasons why optical transduction methods have attracted a great deal of interest and research effort become clear on considering this biosensor format. In general, there is a small number of bioreceptors on a transducer's surface. This means that measurement of analyte binding to a bioreceptor has to be performed with high sensitivity. In addition, the detection technique should only allow for the sensitive detection of molecules on or very close to the surface. Such a surface-specific analyte detection methodology can eliminate interference from the bulk solution, which is particularly important when a bioassay involves a small number of washing steps. The requirements arising from performing measurements in aqueous solutions further limits the suitability of available detection techniques.

Nondestructive detection of real-time kinetic information is also important in the selection of a suitable detection technique. Optical transduction methods are attractive tools that fulfill these requirements. In addition, optical transduction can utilize many different types of spectroscopic techniques, and thereby provides a wide range of data, such as information on the molecular structure and conformation. Most DNA biosensors rely on fluorescence for detection. Laser-induced fluorescence has also been successfully combined with biosensors for ultra-sensitive detection⁸. Conventional photomultiplier tubes and charge-coupled devices (CCDs) are common choices to detect emission of fluorescently-labeled target species. The multichannel capability of CCD makes it very useful for high-throughput detection. However, relatively large, costly and overly complex CCD detection systems are mainly suitable for laboratory analysis. To overcome the size and cost limitations of CCDs, biochips, i.e., integrated biosensor arrays, have been used, where the biochip utilizes a collection of bioreceptor microarrays arranged on a solid

transducer surface that permits many tests to be performed at the same time. The integration and miniaturization leads to higher throughput and speed during biosensing. The advantages of optical transduction methods are also applicable to biochips.

Recently, a novel biosensor based on integrated circuit (IC) microchips with complementary metal oxide semiconductor (CMOS) photocell arrays for optical detection was developed⁹⁻¹¹. The CMOS microchip system is a miniaturized device that integrates photosensors, amplifiers, and logic circuits in a single IC package. Detection of a single bacterium using a sensitive laser-induced fluorescence method has been demonstrated in a CMOS-based biochip system¹².

Our laboratory has developed an integrated circuit (IC) photodiode array (PDA) microchip using conventional bipolar semiconductor technology^{13,14}. The chip can act as a sample platform as well as a two-dimensional photodetector. While the complementary metal oxide semiconductor (CMOS)-based device is driven by voltage, the bipolar PDA chip device is current-driven. This property of the bipolar microchip offers greater advantage for linear amplification of current produced by a photodiode because the output signal of the photodiode is also current. The constructed PDA chip is inexpensive and can provide simultaneous, fast, multifunctional analysis using microdot arrays with a variety of bioreceptors. Successful applications of the bipolar semiconductor technology-based PDA chip for on-chip detection using DNA and protein probe microarrays have been reported^{13,14}. The unique design empowers our chip system with excellent properties such as high-performance analog signal processing at low noise level.

In general, applications of biochips for on-chip bioassays in combination with optical transduction detection methods introduce the need for chemical and biocompatibility of these systems. During the on-chip bioassays, the invariable risk of damage to the PDA detection elements, associated with direct exposure to chemical and biological materials, cannot be avoided. Sequential on-chip bioassays may induce poor signal processing at the wet photodiode detection elements, finally resulting in higher noise levels. To protect the chips, each PDA element in our chips is coated with a thin layer of silicon oxide or silicon nitride.

In further continuation of our research, in this report we demonstrate the operational stability of the PDA chip and the effects of operational parameters such as solution pH that can lead to false or erroneous data interpretation.

Unlike PCR based techniques immunological methods do not require a cell/spore lysis step for extrac-

tion of DNA or RNA. Although PCR techniques using amplification of target gene obviously provides high sensitivity and selectivity, it is time-consuming to obtain the target gene from pathogenic bacteria. Of many bioassays, the enzyme-linked immunosorbent assay (ELISA) promises highly sensitive, highly selective and more rapid analysis of pathogenic bacteria than nucleic acid-based analyses through whole cell detection¹⁵⁻¹⁸. The high sensitivity of ELISA is based on the large number of fluorescent products produced in an immunological sandwich complex by an enzymatic reaction between a substrate and an enzyme-labeled antibody conjugate.

In this work, we report for the first time the potential to detect the pathogenic bacteria *Escherichia coli* O157:H7 using our bipolar PDA microchip system, which is adaptable to field use, based on the combined use of ELISA and laser-induced fluorescence (LIF) detection. To increase the probability for the irradiating laser beam to probe and excite the sample in a small volume, ELISA was performed in an antibody-immobilized capillary reactor. This work shows that the PDA microchip system can also be used as a highly compact and inexpensive alternative to CCD detection for capillary-based reactors.

Results and Discussion

PDA Chip Device

The PDA chip was fabricated using conventional bipolar semiconductor technology. Figure 1 shows a schematic diagram of the photodiode element of the constructed chip. To induce a higher leakage current, in addition to the p-n junction, another n-type impurity (n⁺) was doped into the n-type silicon. This increased the efficiency of the photodiode to respond to a wider wavelength range of light. The photocurrent flowed into the current amplifier through a piece of aluminum denoted as first metal. The current amplifier was protected from exposure to ambient light by another piece of aluminum (the second metal). Each photodiode was also coated with a thin layer of silicon nitride or silicon oxide^{13,14}. The PDA chip and the addressing circuits were described in detail in our previous work^{13,14}. Briefly the PDA chip consisted of a 12 × 12 array of photodiodes, an array of current amplifiers, and a photodiode element addressing circuit that were integrated into a single IC chip. The dimensions of each photodiode were 300 × 300 μm² and the photodiode-to-photodiode distance was 100 μm. A 4 × 12 pin connector, located on the back edge of the PDA chip, was used to transfer the signal from the PDA chip to a test board. The

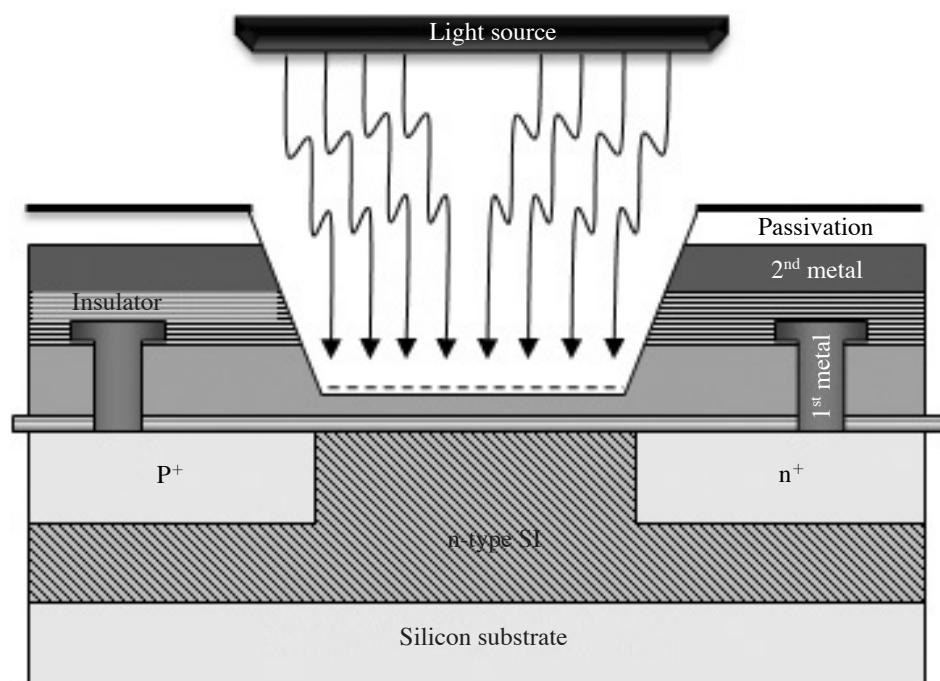


Figure 1. Schematic diagram of the constructed bipolar PDA microchip system. On-chip bioassays were performed directly on the surface of the photodiode detection element.

test board consisted of a TTL logic circuit, an analog-to-digital converter, and a power supply circuit. The microchip detection elements were individually addressed and read out using digital I/O lines and an analog-to-digital conversion channel provided by RS232 interface card installed in a desktop computer. The data acquisition process was controlled via a custom made software interface written in C language.

Photo Response of the PDA Chip

The most important property of the bipolar PDA microchip is an appropriate and sensitive photore-sponse proportional to the light intensity. The system consists of an array of light emitting diodes (LEDs), installed right above the PDA microchip (Figure 1). Generation of photo signals, upon irradiation with a red beam of light are directly proportional to the intensity of red light passing toward the photo detector. Likewise, any hindrance that absorbs the red light decreases the photo signal. This concept has been employed to investigate the variation in photo response caused by any target molecule capable of modulating the red light intensity passing toward the photodetector.

The initial investigation was made to determine the photo-response of the PDA Chip to varying concentrations of methylene blue. Aqueous solution of methylene blue was directly spotted on the PDA chip and the chip was irradiated with a beam of red light. Deionized water was treated as control. The average

output signals from three different detection photodiodes, each spotted with methylene blue solution, were collected at each concentration. Then, the output signal values at the same concentration were averaged for four different chips and the relative reduction in output signal (ΔS) was calculated as follows:

$$\Delta S = \frac{S_0 - S_x}{S_0} \times 100$$

where S_0 is the average output signal of the control, and S_x is the average signal for a sample of concentration x M.

In Figure 2, the percentage change in output signal is plotted as a function of methylene blue concentration. The method is based on the fact that the reduction of photosignal is directly related to the concentration of methylene blue in the spotted sample under a given set of conditions. Photodiode elements covered with methylene blue are screened from the irradiated red light. The extent of screening will be proportional to the amount of methylene blue spotted as the absorption is proportional to the concentration of the sample. Therefore the signal for control sample will always be higher than that for other samples, provided the spot volume is kept constant. Under these test conditions, an excellent linear relationship was obtained over a range of methylene blue concentrations from 10^{-6} to 10^{-4} . The curve in Figure 2 represents best fit of the % change in fluorescence

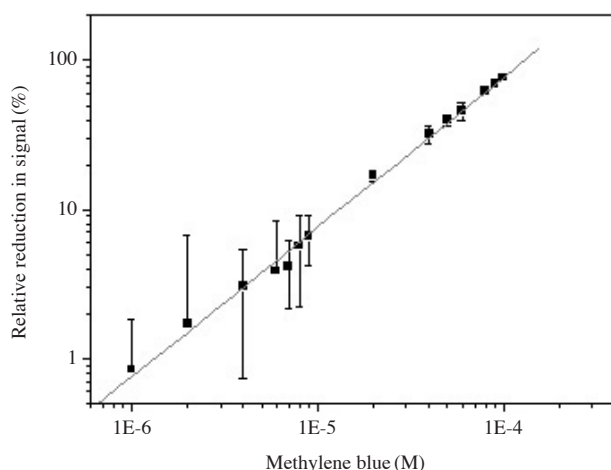


Figure 2. Photo response of the PDA chip, spotted with methylene blue, to red light. Methylene blue solutions of different concentrations were directly spotted on PDA chip (3 pixels/spot). The Y-axis corresponds to the relative reduction in signal with respect to the intensity of the light reaching the photodiode covered with deionized water. As the concentration of methylene blue solution increased, the photodiode signal gradually decreased, i.e., the relative reduction in signal increased (Mean $\pm$ S.D.).

data to a linear equation plotted in a log-log scale ($P < 0.0001$, $R^2 = 9978$).

Stability of Photodiodes over a Series of On-chip Assays for DNA Detection

The probe DNA was immobilized directly on the poly-L-lysine-coated positively charged silicon nitride/oxide layer. The dioxigenin (DIG)-labeled target DNA was then hybridized to the probe DNA. An anti-DIG alkaline phosphatase conjugate was reacted with the hybridized DIG-labeled DNA. A color reaction was performed based on the enzymatic reaction between nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) staining solution and a DNA complex containing antibodies. Consequently, a blue precipitate was produced in proportion to the amount of immobilized DNA. Figure 3 shows a photo image of the PDA chip taken after the DNA hybridization and enzymatic reaction were performed. As a result of the coloring reaction, blue precipitates spread over the detection photodiodes, where the DIG-labeled DNAs were spotted (Figure 3a). The DNA concentrations were prepared by one-quarter serial dilutions from the stock DNA solution, and spotted in four different areas as shown in Figure 3b. When the PDA chip was irradiated with light, the photodiode covered with blue precipitate produced a reduced photocurrent. The enzymatic coloring reac-

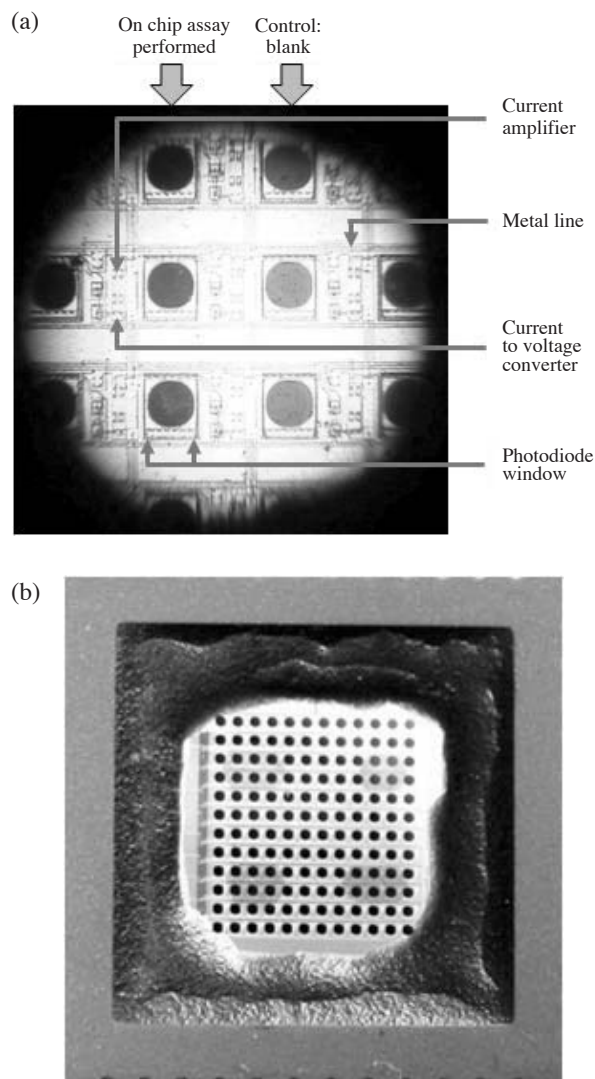


Figure 3. Photo images of a PDA chip taken after the full on-chip bioassay was performed on the surface. (a) The surfaces of the photodiode elements on which enzymatic reactions were performed are covered with the colored product of enzymatic reactions. (b) The DNA stock solution (40 ng/ μ L) was serially diluted to one-quarter the initial concentration. Four serially diluted DNA solutions were spotted on the photodiodes. The blue color corresponds to the blue precipitate produced as the result of the enzymatic reaction between NBT/BCIP and the anti-DIG-alkaline phosphatase conjugate.

tion is obviously a powerful detection method, because it allows easy and rapid access to the results obtained from visualization¹⁹⁻²². However, quantitative analysis is limited by the coloring reaction. Although the coloring reaction provides rapid, easy access to the results, the optical density is proportional to amount of immobilized DNA, which is not

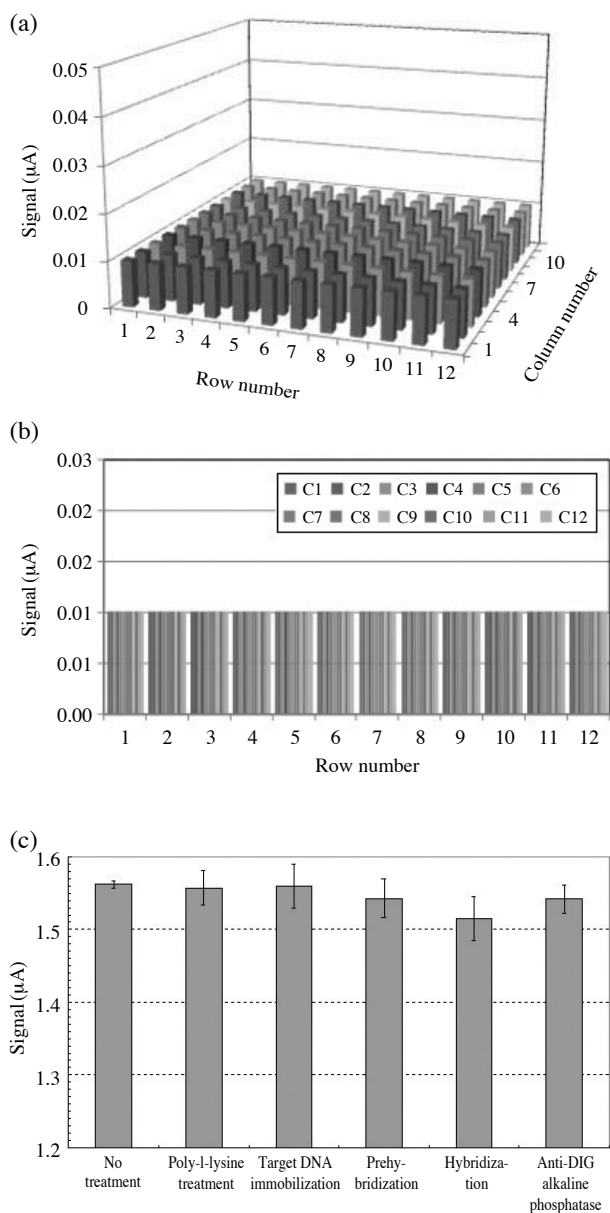


Figure 4. The dark-current stability of the PDA Chip. Signals of all the photodiodes remained almost constant at a very low value. (a) 3D representation, (b) 2D representation, (c) Variance in the background signal of a photodiode at each reaction step of the on-chip bioassay.

enough to provide a precise quantitative result. Thus, only an approximate range of quantitative results can be obtained with the coloring reaction.

The disadvantages of the coloring reaction can be compensated for using a microchip device. If the coloring reaction is performed on a PDA microchip, then a precise quantitative result can be obtained from the digital readout provided by the PDA micro-

chip after light absorption. Complementary to the coloring reaction, which rapidly reveals an approximate range, the digital output of the microchip based on the light absorption of the colored precipitates can provide precise quantitative data from the immobilized target DNA. Our bipolar microchip system demonstrates great potential as a miniaturized portable DNA chip, and is suitable for an on-chip bioassay.

To obtain an output signal for four different concentrations from the chip, the average output signal from four different detection photodiodes was collected at each concentration; this was repeated for four different chips. Then, the output signals of four different chips were averaged at each concentration, and the output signal was plotted versus concentration. Figure 4a and b show the variance in the dark-current signal of the bare chip. This result shows the stability in the noise level. In addition, the standard deviation was within 10% for the output signals obtained from four different chips under identical conditions, which reflects the consistent properties of the fabricated PDA chips.

The stability of the PDA chip over the entire on-chip bioassay was investigated by observing the variance in the output voltage of a photodiode exposed to the reaction solution at each reaction step; the data is shown in Figure 4c. The background signal of the PDA chip remained almost unchanged over the entire reaction period. The error bars in Figure 4c represent the variance in signals obtained from 12 photodiodes at each reaction step. The standard deviation in the signals from the 12 photodiodes at each reaction step was less than 5%.

Effect of Solution pH on the Photoresponse

On-chip bioassays involve multiple reactions and washing steps. During an on-chip assay the photodiode is directly exposed to bulk solutions and bio-reagents. Therefore, to minimize the possibility of damage to the photodiode, it is absolutely necessary to optimize reaction conditions. Simultaneously, the optical transduction efficiency of PDA elements should also be considered. To protect the PDA elements in our microchip, each photodiode was covered with a layer of silicon nitride (Si_3N_4) or silicon oxide. In addition, the pHs of the solutions were purposefully manipulated to pH 7-7.5. However, even under optimized conditions, an on-chip bioassay may still cause a certain level of damage to the photodiode due to unavoidable interference from the bulk medium. The situation may become worse if the pH of the solutions cannot be controlled precisely. This becomes particularly important for a PDA chip that is intended for repeated use. Therefore, it is desirable to

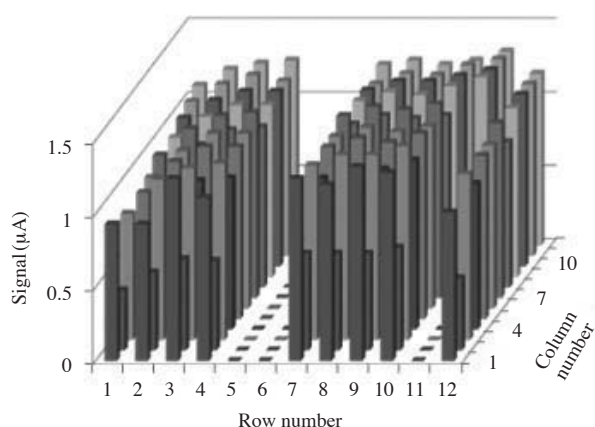


Figure 5. Effect of harsh reaction conditions on the photo response.

monitor the operational capacity of the chip at each reaction step. At the same time, it is important to carefully analyze the data as consideration of false data may lead to erroneous results. Figure 5 is an example of an erroneous result. Here, some of the photodiodes were damaged due to either the harsh reaction conditions or mishandling. The signals in rows 5, 6 and 11 were abnormally low compared to other photodiode signals. Hence, if the data were considered column-wise, a definite misinterpretation would result. There are also small variations in the signals of other photodiodes. But, since there are 12 photodiodes in a row or column, and an average of 12 data points are considered from each row or column, this small variation in individual signals will not significantly affect the average output for a particular bioassay.

Detection of *E. coli* O157 : H7 based on ELISA

Immunological methods are among the most promising techniques for selective identification of biological agents²³. This becomes particularly important when low numbers of pathogenic species coexist with non-pathogenic organisms in a complex biological environment. The most attractive features of immunological techniques are the specific interaction between capture probes and target biomaterials and the capability for field use²⁴. Unlike nucleic acid-based analyses such as polymerase chain reaction (PCR), immunological methods do not require the cell/spore lysis step used for the extraction of DNA or RNA. This is especially advantageous in detecting bacterial spores¹² that have disruption-resistant durable shells. Therefore, immunological methods can

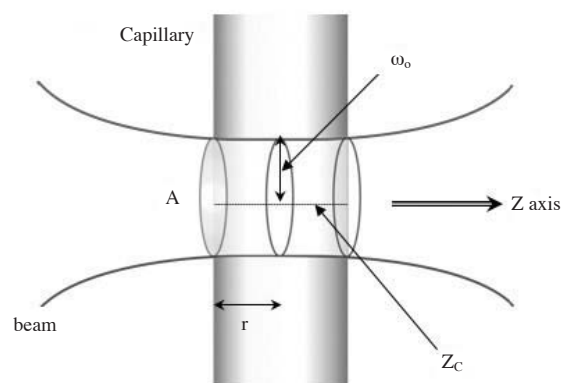


Figure 6. Schematics of a capillary irradiated with a laser beam.

achieve much more rapid detection of biological agents than nucleic acid-based methods. In addition, the enzyme-linked immunosorbent assay (ELISA) can further enhance detection sensitivity due to its unique ability to amplify enzymatic products.

Conventional sample platforms such as zeta probe membrane or glass are not compatible when ELISA is performed in solution, as the fluorescence emission from one ELISA unit cannot be isolated spatially from another. Conventional multi-well plates, which can provide well-isolated reaction chambers for ELISA, are not size-compatible with miniaturized biochip systems for use as sample platforms. However, a capillary reactor, optically coupled to a biochip, could be an excellent solution to this problem.

Laser beam irradiation is an appropriate optical configuration for the biochip system due to its simplicity and compactness. The advantage of this excitation geometry is that the biochip can be placed perpendicular to the laser scattering thereby minimizing the noise level. Our PDA microchip pixel is comparable to the outer diameter of a single capillary reactor (365 μm). Based on this condition, the optical correspondence between the capillary reactor and a photodiode pixel was accomplished with the magnification of the single capillary reactor image using a 5× microscope objective. By spatial adjustment of the biochip using a three-dimensional translational stage, the fluorescence image of ELISA from the single capillary reactor could be easily detected at the highest signal-to-noise ratio.

Under particular optical conditions, LIF has a theoretical potential for the detection of a single fluorophore [42]. In addition, fused silica capillary increases the probability for irradiated laser beam to probe and excite a sample in the small volume of the capillary. As shown in Figure 6, the confocal distance (Z_c)

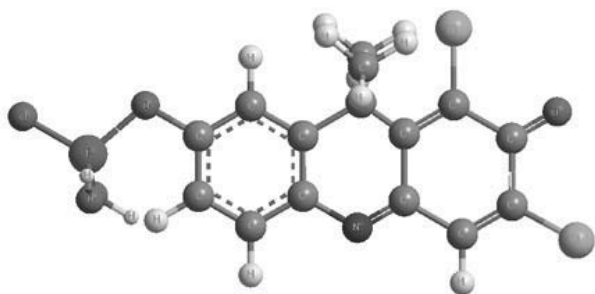


Figure 7. Structure of DDAO-phosphate 9H-(1, 3-dichloro-9, 9-dimethylacridin-2-one-7-yl)-phosphate.

can be calculated according to the equations used by Song *et al.*^{12,25}.

$$Z_c = \frac{-2z + \sqrt{4z^2 - 4\left(1 - \frac{A_z}{\pi\omega_0^2}\right)z^2}}{2\left(1 - \frac{A_z}{\pi\omega_0^2}\right)}$$

$$\omega_0 = 1.22\lambda \cdot \frac{f}{D},$$

where ω_0 is the radius of the focused beam in the focal region, A is the beam area, z is the beam propagation axis, f is the focal length of the lens, λ is the wavelength of the beam, and D is the beam diameter.

The laser probe volume (V) can be calculated as

$$V = \int_0^r A_z dz = \pi \frac{\omega_0^2}{\lambda}$$

where r is the inner radius of the capillary, $\pi\omega_0^2/\lambda$ ²⁶ and V is the laser probe volume.

Based on the above equations, in a hypothetical case, with a lens with a focal length of 41 mm and a beam diameter of 2 mm, the laser probe volume is calculated to be as low as 80 pL.

In this study, due to the amplification of fluorescence emission highly sensitive detection of *E. coli* O157:H7 was achieved. The non-fluorescent DDAO-phosphate (Figure 7) produced a low background signal. On the other hand one alkaline phosphatase cleaved many non-fluorescent DDAO-phosphates, and a large amount of cleaved fluorescent DDAO was produced.

Figure 8 shows the fluorescence detection of DDAO cleaved from sandwich complexes by ELISA in the capillary reactor. The concentration of *E. coli* O157:H7 and alkaline phosphatase conjugate was 7×10^6 cells/mL. Only one photodiode element in one column, optically coupled with the capillary reactor, showed significant optical response, while the other photodiode elements had dark current signals. Each

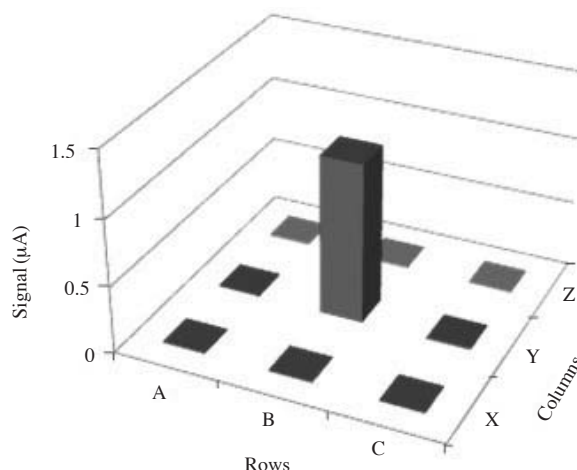


Figure 8. Demonstration of ELISA using a capillary reactor-biochip system. The higher fluorescence intensity corresponds to the ELISA while the lower fluorescence intensity corresponds to the background signal.

immunoassay had a control that did not have any *E. coli* O157:H7. This control corresponded to the background signal. A detection limit as low as 10 cells/mL was achieved by this method (data not shown).

Conclusions

In this work, we demonstrated the potential of a portable miniaturized bipolar biochip system for the detection of biomolecules both on-chip and captured in an antibody-based capillary reactor. The effects of operational parameters like pH on the stability of PDA elements were also investigated. Capillary-based detection was achieved through enzymatic amplification of alkaline phosphatase conjugated to antibody and DDAO-phosphate substrate. This enzymatic amplification eliminated the need to use a complicated and bulky optical system to achieve high sensitivity levels. This portable biochip system is expected to contribute significantly to environmental sensing and medical diagnosis.

Materials and Methods

Materials

Tris (hydroxymethyl)amino (Tris) used was purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Ethidium bromide and 9H-(1, 3-dichloro-9, 9-dimethylacridin-2-one-7-yl)-phosphate, diammonium salt

(DDAO-phosphate) were purchased from Molecular Probes (Eugene, OR, USA). The enzyme, buffer, and dNTP mixture used for PCR were purchased from Applied Biosystems (Foster City, CA). The DIG DNA labeling and detection kit was purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). Methylene blue, phosphate-buffered saline (PBS), (3-aminopropyl)triethoxysilane (APTES), and toluene were purchased from Sigma. Glutaraldehyde was obtained from Electron Microscopy Sciences (Fort Washington, PA, USA). Fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA). Heat-killed *E. coli* O157:H7, anti-*E. coli* O157:H7 antibody (Catalog No. 01-95-90), alkaline phosphatase-labeled anti-*E. coli* O157:H7 antibody (Catalog No. 05-95-90), and blocking agent/BSA solution were purchased from Kirkegaard & Perry Labs. (Gaithersburg, MD, USA)

PCR Protocol, Dioxigenin DNA Labeling and DNA Hybridization

The *Bacillus subtilis* *sspE* gene, the target gene, was amplified by PCR. Two primers, *sspE*-1, 3'-AGGAATAGCTATACGATCAC-5' and *sspE*-2, 3'-AGTGCTTTTTTCTGTTTCAG-5' were used to obtain the 340 bp PCR product. Details of the PCR protocol, dioxigenin labeling and DNA hybridization

protocols were described in our previous work¹³.

Instrumentation for Fluorescence Detection

Figure 9 shows a schematic diagram of the PDA microchip detection system for laser-induced fluorescence-based monitoring of ELISA. A compact semiconductor laser beam at 635 nm (Edmund Industrial Optics, Barrington, NJ, USA) was used as the excitation source to excite the fluorescent dye DDAO. The diode laser beam was focused onto a capillary reactor using a 5× microscope objective (Nikon, 0.1 NA). The capillary reactor was fixed to a capillary holder. The vertical displacement of the capillary was controlled by an *x-y* translational stage. The detection window of the capillary reactor was made by removing the polyimide coating. The fluorescence emission from the capillary reactors was collected using a 5× microscope objective (Nikon, 0.1 NA) and focused onto a photodiode detection element. A narrow bandpass filter (central wavelength: 656 nm, FWHM: 10 nm, Edmund Industrial Optics) was placed in front of the bipolar microchip to remove any laser scattering.

ELISA

First a fused-silica capillary column (10 cm length and 75 μm i.d.) was treated with 10% (v/v) (3-amino-

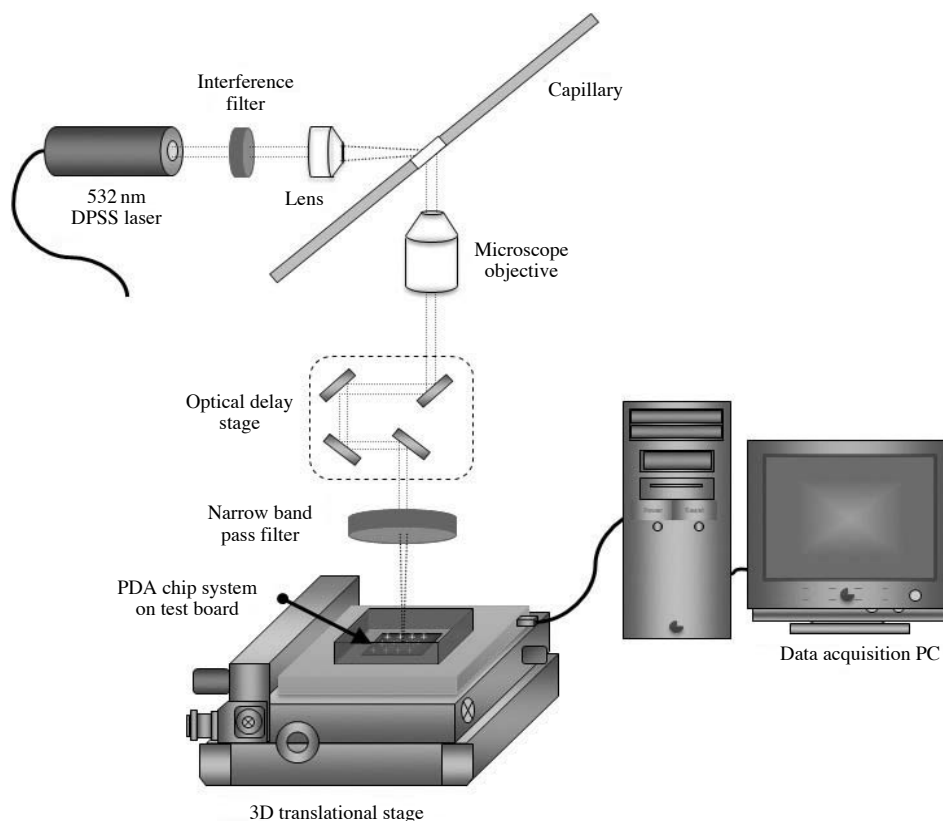


Figure 9. Schematic diagram of the constructed capillary reactor-biochip system.

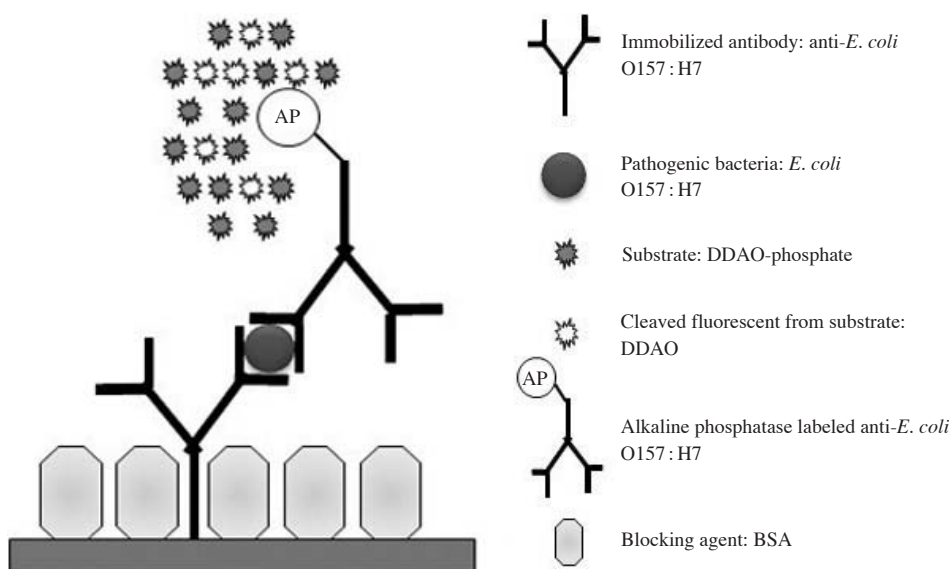


Figure 10. Schematic diagram of immunoassay (ELISA) performed in the capillary reactor to detect *E. coli* O157:H7. Cleaved fluorescent products are produced by enzymatic reaction between substrate and sandwich immunocomplex.

propyl)triethoxysilane in dry toluene, followed by heating overnight at 115°C. The excess silane was removed by rinsing with toluene. The capillary was then treated with 2.5% (v/v) glutaraldehyde in phosphate buffer (10 mM, pH 7). Unbound glutaraldehyde was rinsed off with phosphate buffer. As shown in Figure 10 anti-*E. coli* O157:H7 antibodies, specific to a surface antigen on *E. coli* O157:H7, were immobilized on the inner wall of the glutaraldehyde-treated capillary overnight at 4°C. Otherwise, the incubation time for each reaction was 1 h. The anti-*E. coli* O157:H7 was diluted to 10 µg/mL in PBS per the supplier's protocol. After the capture antibodies were immobilized, the capillary was filled with an aqueous BSA diluent/blocking solution for 1 h. The capture antibodies were incubated with *E. coli* O157:H7 diluted to various concentrations in PBS. Following incubation with *E. coli* O157:H7, the antibodies were rinsed with 0.5% Tween 20 in PBS to remove any unbound target *E. coli* O157:H7. Then, alkaline phosphatase labeled anti-*E. coli* O157:H7 antibodies (5 µg/mL), which recognize another epitope on the *E. coli* O157:H7 surface, were incubated with the captured *E. coli* O157:H7. Excess antibodies were washed away with 0.5% Tween 20 in PBS. A 0.05 mM DDAO-phosphate solution was injected to the capillary for fluorescence detection. A 5 mM stock solution of DDAO-phosphate was prepared by dissolving the stock in deionized water. The resulting 5 mM solution was diluted to 0.05 mM with carbonate buffer solution (pH 9). Fluorescence emission was detected immediately after the addition of diluted DDAO-phosphate solution.

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