Miniature biochip system for detection of *Escherichia coli* O157:H7 based on antibody-immobilized capillary reactors and enzyme-linked immunosorbent assay

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Abstract

In this work, we report *Escherichia coli* O157:H7 detection using antibody-immobilized capillary reactors, enzyme-linked immunosorbent assay (ELISA), and a biochip system. ELISA, a selective immunological method to detect pathogenic bacteria, ELISA is also directly adaptable to a miniature biochip system that utilizes conventional sample platforms such as polymer membranes and glass. The antibody-immobilized capillary reactor is a very attractive sample platform for ELISA because of its low cost, compactness, reuse, and ease of regeneration. Moreover, an array of capillary reactors can provide high-throughput ELISA. In this report, we describe the use of an array of antibody-immobilized capillary reactors for multiplex detection of *E. coli* O157:H7 in our miniature biochip system. Side-entry laser beam irradiation to an array of capillary reactors contributes significantly to miniaturized optical configuration for this biochip system. The detection limits of *E. coli* O157:H7 using the ELISA and Cy5 label-based immunoassays were determined to be 3 and 230 cells, respectively. This system shows capability to simultaneously monitor multifunctional immunoassay and high sensitive detection of *E. coli* O157:H7.

Keywords: Biochip; Enzyme-linked immunosorbent assay; *Escherichia coli* O157:H7; Antibody-immobilized capillary

1. Introduction

Bacteria that exist widely in the environment have close relations with humans. The bacteria can spread quickly and easily through water and food under appropriate temperature and moisture conditions. It has been reported that more than 150 kinds of bacteria exist both inside and outside of the human body [1]. Many infectious diseases such as pneumonia, cholera, etc. are caused by pathogenic bacteria, which would be fatal to humans. The fact that infectious diseases are involved in nearly 40% of the total 50 million annual estimated deaths clearly shows the harmfulness of pathogenic bacteria [2]. The number of pathogenic bacteria have been increasingly identified as important food- and water-borne pathogens. *Escherichia coli* O157:H7 is a representative food-borne pathogen that produces large amounts of a potent toxin in the lining of the intestine.

This can induce hemorrhagic colitis or hemolytic uremic syndrome which may lead to death, particularly in children [3]. The infective dose of *E. coli* O157:H7 has been estimated to be as low as 10 cells [4]. Immunological methods are among the most promising techniques for detection and species-specific identification of food-borne pathogenic bacteria. Selective identification of specific bacteria is very important because there are many situations where low numbers of pathogenic bacteria coexist with large numbers non-pathogenic organisms in a complex biological environment. The immunological methods do not require a cell/spore lysis step used for the extraction of DNA or RNA. Although the PCR technique using amplification of target gene obviously provides high sensitivity and selectivity, it is a time-consuming process to obtain the target gene from pathogenic bacteria. On the other hand, immunological methods can promise more rapid analysis of pathogenic bacteria than nucleic acid-based analyses through whole cell detection. Of immunological methods, enzyme-linked immunosorbent assay (ELISA) is a very powerful tool that provides high sensitivity as well as high selectivity [5–8].
The sensitivity is based on amplification of fluorescent product by an enzymatic reaction between a substrate and an enzyme-labeled antibody conjugate. A large number of fluorescent products can be produced at each immunological sandwich complex, which leads to high sensitivity. Biochip systems compose a special class of biosensors based on integrated circuit microchip and multiple transducer elements. One of the advantages of a biochip system is its capability to simultaneously monitor multifunctional bioassays using sample substrates having microarrays of bioelements. Recently, our laboratory has developed a miniaturized biochip system that consists of both integrated microsensor detection systems and microarray of probes used for biochemical assays[9–13]. This entire system is compact and portable enough to be readily adapted from laboratory-based research to field use. ELISA is a very appropriate bioassay for use with the miniaturized biochip system due to its high sensitivity and selectivity. However, some further development of a miniaturized appropriate sample platform to accommodate ELISA is required. Solid supports such as polymer membranes and glass have been used as representative sample platforms in the biochip system. ELISA is directly adaptable to these sample platforms. In these sample platforms, insoluble assay product (i.e. precipitable product from the ELISA assay) can be produced at localized sites on the membrane or glass surface. However, when a soluble enzymatic product is produced from the ELISA reaction, as is the case in this present study, then some form of spatial isolation for each assay must be used. Accordingly, a certain sample platform to isolate and discriminate each ELISA is absolutely necessary in order to increase the multiplex capability of ELISA in the biochip system.

In this paper, we report the use of an array of antibody-immobilized capillary reactors as a sampling platform for ELISA in biochip system to detect E. coli O157:H7. The antibody-immobilized capillary reactors are compact, inexpensive, and readily adaptable to microfluidic systems [14–16]. Each capillary reactor can isolate its ELISA fluorescence emission from other capillary reactors. As a result, high throughput of well-isolated ELISA can be achieved. In addition, the small radius of each capillary reactor provides a small detection volume. In this manuscript, it is shown that ELISA based on an array of antibody-immobilized capillary reactors provides both high sensitivity and a multiplex bioassay capability to the biochip system for the detection of pathogenic bacteria.

2. Materials and methods

2.1. Materials/chemicals

Phosphate buffered saline (PBS), (3-aminopropyl)-tri-ethoxysilane (APTES), and toluene were purchased from Sigma. 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)phosphate, diaminonitro salt (DDAO-phosphate) was purchased from Molecular Probes (Eugene, OR, USA). Glutaraldehyde was obtained from Electron Microscopy Sciences (Fort Washington, PA, USA). Fused-silica capillary columns were purchased from Polymicro Technologies (Phoenix, AZ, USA). Anti-E. coli O157:H7 (Catalog No. 01-95-90) and alkaline phosphatase labeled anti-E. coli O157:H7 (Catalog No. 05-95-90) were obtained from Kirkegaard & Perry Labs. (Gaithersburg, MD, USA). Goat anti-E. coli O157:H7 was purchased from Biodesign International (Catalog No. B651098, Saco, ME, USA). Rabbit anti-sheep IgG(H+L) Cy5 conjugate (Catalog No. 81-8616) was obtained from Zymed Labs. (San Francisco, CA, USA).

2.2. Instrumentation

Fig. 1 shows a schematic diagram of the integrated circuit microchip detection system for laser-induced fluorescence-based monitoring of immunoassays. The complementary metal oxide semiconductor (CMOS) technology was used to fabricate our microchip that consists of a two-dimensional 4 × 4 array of photodiode detection elements. The physical dimension of each photodiode element is 900 μm × 900 μm and the distance between two photodiode elements is 100 μm. The CMOS microchip was custom-designed in Oak Ridge National Laboratory. Advantages of CMOS production include low-power consumption and low-cost fabrication. Another benefit of CMOS photosensors is the high level of product integration that can be accomplished through implementing virtually all of the electronic sensor functions onto the same integrated circuit (IC) chip. CMOS technology is very appropriate for this integration. Exploiting these advantages, our CMOS microchip system was fabricated to integrate the logic circuitry, amplifiers, and discriminators along with the photodiode detection elements. This integration led to a compact CMOS microchip system. The individual photodiode detection elements in the CMOS microchip were accessed and read out using the digital I/O lines and the analog-to-digital conversion in a National Instruments DAQ516PCMCIA card installed in a Laptop computer. Special software (Labview platform) was used to control CMOS microchip operation. As shown in Fig. 1, a compact semiconductor laser beam at 635 nm (Edmund Industrial Optics, Barrington, NJ, USA) was used as an excitation source to excite the
fluorescent dyes, Cy5 and DDAO. The diode laser beam was focused onto an outermost capillary reactor of an array of four capillary reactors. The four capillary reactors were packed side by side and held by a X-Y translational stage. The detection windows of the four capillary reactors were made by stripping their polyimide coatings. In order for the laser beam to pass through all detection windows of the four capillary reactors, the vertical displacement of the four capillary reactors was controlled by the X-Y translational stage [17–20]. The fluorescence emission from the four capillary reactors was collected using a 5× microscope objective (Nikon, 0.1 NA) and focused onto four photodiode detection elements. A narrow bandpass filter (central wavelength: 656 nm, FWHM: 10 nm, Edmund Industrial Optics) was attached to the CMOS microchip to reduce scattering.

3. Immunoassays

For the immobilization of antibodies, fused-silica capillary columns of 10 cm length and 100 μm i.d. were treated with 10% (v/v) (3-aminopropyl)triethoxysilane in toluene using a syringe, followed by heating overnight at 115°C. Then, capillaries were treated with 2.5% (v/v) glutaraldehyde in phosphate buffer of pH 7 for the immobilizations of antibodies. Two immunoassays were attempted. Alkaline-phosphatase labeled antibodies were used for ELISA while Cy5-labeled antibodies were used for fluorescence emission without amplification. As shown in Fig. 2a, anti- E. coli O157:H7 antibodies specific to a surface antigen on E. coli O157:H7 were immobilized onto the inner wall of capillary overnight at 4°C. Otherwise, incubation times for each reaction were 1 h. The anti-E. coli O157:H7 was diluted to 10 μg/ml in PBS. After the capture antibodies were immobilized, the capillaries were filled with a BSA diluent/blocking aqueous solution for 1 h in order to block the remaining binding sites of the inner wall. Then, 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 eliminate the unbound target E. coli O157:H7. After the rinse, alkaline phosphatase labeled anti-E. coli O157:H7 antibodies of 3 μg/ml, which recognize another epitope on the E. coli O157:H7 surface, were incubated with the captured E. coli O157:H7. Following a rinse step with 0.5% Tween 20 in PBS, a 0.05 mM DDAO-phosphate solution was added to the sandwich complex for fluorescence detection. A 5 mM stock solution of DDAO-phosphate was prepared by dissolving the stock solution into deionized water. The resulting stock solution was diluted to 0.05 mM with carbonate buffer solution of pH 9. Fluorescence emission was immediately detected after the diluted DDAO-phosphate solution was added. As shown in Fig. 2b, another fluorimunooassay without the amplification of fluorescent product was performed. Identical capture antibodies used in ELISA were immobilized and E. coli O157:H7 was captured onto the capture antibodies. Then, the detector antibody, goat anti-E. coli O157:H7, was diluted 1:50 in PBS and was incubated with the captured E. coli O157:H7. Following a rinse step with 0.5% Tween 20 in PBS, the final antibody, a purified rabbit anti-goat IgG(H + L) antibody conjugated with Cy5, was diluted 1:50 in PBS and was incubated with the sandwich complex to complete the immunoassay.

3. Results and discussion

ELISA provides extremely high sensitivity due to the amplification of fluorescence emission as a result of the reaction between DDAO-phosphate substrate and alkaline phosphatase conjugate to yield fluorescent products in large quantities. One alkaline phosphatase can actually cleave many non-fluorescent DDAO-phosphate. As a result, a large amount of cleaved DDAO is produced. The non-fluorescent DDAO-phosphate is very advantageous in that it yields low background signal. The conventional sample platforms such as a zeta probe membrane or glass are compatible with ELISA. However, these sample platforms are not adequate for use in multiplex ELISA. This is because the enzymatic fluorescent product from each ELISA is produced in solution, and as a result, the fluorescence emission from one ELISA unit cannot be isolated spatially from another. Although conventional 96-well plates provide well isolated reaction chambers for ELISA, their large overall size is inappropriate for use as sample platforms in the miniaturized biochip systems. One of our solutions to this problem is to use low cost, commercially available capillaries as reaction chambers. There are several reasons for this approach. First, both the submicro-liter range of sample volume and the light transparency of the capillary reactor are directly adaptable to the miniaturized biochip system. Second, antibody immobilization on glass surface using glutaraldehyde is a well-established technique for antigen capture and can be adapted to the capillary reactor easily. Furthermore, in an array of capillary reactors, each individual capillary reactor can be used as a reaction chamber unit of ELISA and be optically and spatially isolated from other capillary reactors if an appropriate optical configuration is used. Moreover, capillary reactors are reusable and their regeneration is relatively easy. Based on these properties, the use of an array of capillary reactors as a sample platform, which provides multiplex capability for ELISA, is a promising solution.

As shown in Fig. 1, side-entry laser beam irradiation was used to simultaneously detect fluorescent products of ELISA at four capillary reactors. This side-entry laser beam irradiation was an appropriate optical configuration for the biochip system due to its simplicity and compactness. This excitation geometry precludes the need for a mechanical scanning system, as is needed for confocal scanning microscopy. Another advantage of this side-entry laser beam irradiation is that the biochip can be placed perpendicular to the laser scattering.
As a result, background signal can be minimized significantly. The effectiveness of side-entry laser beam excitation is dependent on how many capillary reactors the laser beam passes through, while each individual capillary reactor acts as a lens. The reduction of laser beam intensity in this optical configuration mainly occurs due to reflection and refraction at the boundaries of capillary reactors that cause the change of refractive index. When an array of capillary reactors filled with aqueous solution is placed in air, capillary reactors with ratios of inner diameter to outer diameter of less than 6 and more than 2 can act as a convex lens, hence the incident laser beam can be refracted to the central axis of capillary reactor and pass through all detection windows of capillary reactors [21]. Accordingly, the capillary reactors with ratios of inner diameter to outer diameter of 3.6 were used for side-entry laser beam irradiation in this study. Another important challenge in the construction of our miniature biochip system was to achieve appropriate optical coupling between the photodiode detection elements and the capillary reactors. The detection elements of our microchip have large physical dimension (900 μm × 900 μm) when compared to the pixel dimension of a conventional charge-coupled device (CCD) detector (typically 20 μm × 20 μm). Our CMOS microchip pixel is even larger than the outer diameter of a single capillary reactor (365 μm). Based on this condition, the optical correspondence between the capillary reactor and the CMOS microchip pixel was accomplished with the magnification of single capillary reactor image. A 5×
Fig. 3. (a) Simultaneous detection of E. coli O157:H7 at four capillary reactors using the ELISA. The concentrations of E. coli O157:H7 were identical, $7 \times 10^6$ cells/ml at four capillary reactors. (b) Demonstration of the multifunctional bioassays using the capillary reactors–biochip system. The higher fluorescence intensity corresponds to the ELISA while the lower fluorescence intensity corresponds to Cy5 label-based immunoassay. Two immunoassays have identical concentrations of E. coli O157:H7 ($7 \times 10^6$ cells/ml). Two controls mainly show the laser scattering: (row number 2, column number 1) ELISA; (row number 2, column number 2) control of ELISA; (row number 2, column number 3) immunoassay using a Cy5-labeled antibody; (row number 2, column number 4) control of Cy5 label-based immunoassay.

The microchip system was held by a translational stage which moves in three dimensions. Through spatial adjustment of the biochip using this three-dimensional translational stage, the fluorescence images of ELISA from four capillary reactors could be detected with the highest signal-to-noise ratio. Fig. 3a shows the simultaneous fluorescence detection of DDAO cleaved from sandwich complexes by ELISA in four capillary reactors. The concentrations of E. coli O157:H7 and alkaline phosphatase conjugate was $7.4 \times 10^6$ cells/ml and $5 \times 10^{-5}$ M, respectively. Only four photodiode elements in one column, optically coupled with four capillary reactors, show optical response while other photodiode elements show dark current signals. The well-defined correspondence between the optical response of each photodiode element and each respective capillary reactor demonstrates the suitability of an array of capillary reactors as a sample platform, allowing isolated detection of each ELISA reaction. The multiplex monitoring of ELISA could be achieved by this array of four capillary reactors that prevent spatial overlap of ELISA fluorescence emissions from each ELISA reactor. However, there is reduction of laser beam intensity due to the
ometry described above and the large distance (100 μm) between two pixels contributed to minimal exposure of each photodiode detection element to stray light from adjacent capillaries. As a result, the cross-talk was negligible in our photodiode detection element to stray light from adjacent capillaries. As a result, the cross-talk was negligible in our microchip. Fig. 3b shows simultaneous detections of E. coli O157:H7 using two different immunoassays. The higher fluorescence intensity (row number 2, column number 1) corresponds to ELISA and the lower fluorescence intensity (row number 2, column number 3) corresponds to an immunosassay using a Cy5-labeled antibody. The concentration of E. coli O157:H7 was 7.4 × 10^6 cells/ml. Each immunoassay has a control (row number 2, column number 2 and row number 2, column number 4) that does not have any E. coli O157:H7. This control showed the background signal. The fluorescence intensity of immunosassay based on Cy5-labeled antibodies was much smaller than that of ELISA. The lower fluorescence intensity clearly demonstrates the advantage of using ELISA with the amplification of fluorescent product for higher sensitivity. Also, this figure represents the capability of a capillary reactor–microchip system to simultaneously monitor multifunctional immunoassays.

Fig. 4 shows a quantitative comparison of two fluoroimmunoassays. The upper line corresponds to fluorescence intensities of ELISA obtained at a single capillary reactor as a function of number of E. coli O157:H7. On the other hand, the lower line corresponds to fluorescence intensities from the Cy5-labeled antibody-sandwich complex. The estimated number of organisms probed for each measurement was based on the calculation of the inner volume of the 10 cm capillary, assuming even distribution of organisms inside the capillary. To obtain each data point in Fig. 4, triplicate measurements were performed and the average value was plotted. The fluorescence intensity obtained using ELISA was at least 10-fold higher than that obtained using the Cy5-labeled antibody-sandwich complex at identical number of E. coli O157:H7. Compared to the immunosassay without any amplification of fluorescent product, each E. coli O157:H7 can produce a lot of cleaved fluorescent DDAO in ELISA by the enzymatic reaction between the substrate and the alkaline phosphatase conjugate. This amplification contributed significantly to the higher E. coli O157:H7 detection sensitivity of ELISA. The detection limits (at S/N = 3) of E. coli O157:H7 using the ELISA and Cy5 label-based immunoassays were 3 and 230 cells, respectively. In this case, noise was defined as the standard deviation of the background obtained when the bioassays were performed without E. coli cells. Note that these detection limits can be improved when the biochip system is connected to a flow-through system such that bacteria in the sample stream will be continuously collected at the immobilized capture antibody sites in the capillary reactor.

4. Conclusion

High-throughput ELISA for the detection of E. coli O157:H7 was attempted using an array of four capillary reactors in a biochip system. An individual antibody-immobilized capillary reactor could be successfully used as a reaction chamber for isolated ELISA. Side-entry laser beam irradiation to an array of four capillary reactors permitted the simultaneous excitation of fluorescent ELISA products at four capillary reactors and was optically compatible with the miniature biochip system. Also, our biochip-multiple capillary reactor system showed feasibility for simultaneous monitoring of multiple immunoassays.

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