Articles

Microfluidic Immunoassay Platform Using Antibody-immobilized Glass Beads and Its Application for Detection of *Escherichia coli* O157:H7

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We developed a microfluidic immunoassay platform for the detection of various analytes such as bacterial pathogen by packing antibody-immobilized glass beads in spatially-isolated microchambers on a microfluidic device. Primary amines of antibody were covalently conjugated to carboxyl-terminated glass beads previously treated with aminosilane followed by glutaraldehyde. Through this covalent binding, up to 905 μg immunoglobulin G (IgG) per gram of glass beads was immobilized. For application, glass beads attaching antibody specific to *Escherichia coli* O157:H7, a foodborne pathogen, were packed into a microfluidic device and used for the detection of the serotype. This prototype immunoassay device can be used for the simultaneous detection of multiple analytes by sequentially packing different-sized glass beads attaching different antibody in discrete microchambers on a single microfluidic device.

Key Words : Microchamber, Glass bead, IgG immobilization, Pathogenic bacteria, *Escherichia coli* O157:H7

Introduction

Immunoassays are powerful bioanalytical systems which utilize the sensitivity and specificity of the antibody-antigen interaction. However, immunoassays often suffer from long analysis time, high cost, and lack of automation. To overcome these problems, many efforts have been focused on miniaturization of the immunoassay system using microfluidic technology because the technology can decrease analysis time, reduce the consumption of expensive reagents, and precisely control the movement of fluids.[1] Especially heterogeneous immunoassays, which require the immobilization of antibody on a solid support, have been extensively studied on microfluidic devices because their intrinsic property of higher surface area to volume ratio can increase binding chance between antigen and antibody. The surface area in microfluidic devices can be increased further by fabricating porous structures, packing microbeads, and installing polymeric membranes in microchannels.[1]

Antibody-attached microbeads packed in microchannel have many advantages over the planar surface immobilized with antibody. Microbeads can drastically increase the available surface area inside microchannel and can be easily replaced with a fresh aliquot of beads between assays. Since antibody immobilization on microbeads can be done outside and later packed inside the microchannels, the unnecessary exposure of antibody to unwanted surface in microchannels can be minimized.

In this study, we report a microfluidic immunoassay platform simple in design, fabrication, and operation. In details, glass beads were used as solid support because they are cost-effective and their surface can be easily modified with known conjugation chemistry. Capture antibody specific to *Escherichia coli* O157:H7 (*E. coli* O157:H7), a foodborne pathogen, was covalently immobilized on glass beads *via* amide linkage between amino groups on antibody and carboxyl-terminated surface of beads.[2-5] Due to vigorous hydrodynamic flow generated inside bead-packed microchambers, sufficient mixing which is required for better binding of antigen with immobilized antibody could be achieved, facilitating antibody-antigen binding process and also eliminating extra mixing process. The feasibility of our microfluidic immunoassay platform was studied by investigating the binding specificities of various antibody-antigen reactions and later detecting *E. coli* O157:H7 as a model analyte.

Experimental Sections

Materials. SU-8 2050 was purchased from MicroChem Corp. (Newton, MA). PDMS prepolymer and curing agent (Sylgard 184) were purchased from Dow Corning (Midland, MI). Glass beads with average diameters of 150-212 μm, glutaraldehyde solution (95%), L-lysine (98%), rabbit IgG, goat IgG, fluorescein isothiocyanate (FITC)-tagged anti-rabbit IgG, and FITC-tagged anti-goat IgG were purchased from Sigma (St. Louis, MO). 3-aminopropyl trimethoxysilane (APTMS, 97%),[6] sodium cyanoborohydride (95%), and ninhydrin (97%) were purchased from Aldrich (Milwaukee, WI). Fluorosilane agent, (tridecafluoro-1,1,2,2-tetrahydro-octyl)-1-1-trichlorosilane, was obtained from United Chemical Technologies. Borate buffer was prepared from sodium tetraborate (10 mM, pH 7.0) and phosphate-buffered saline (PBS, 10 mM, pH 7.4) was prepared from 10 mM sodium

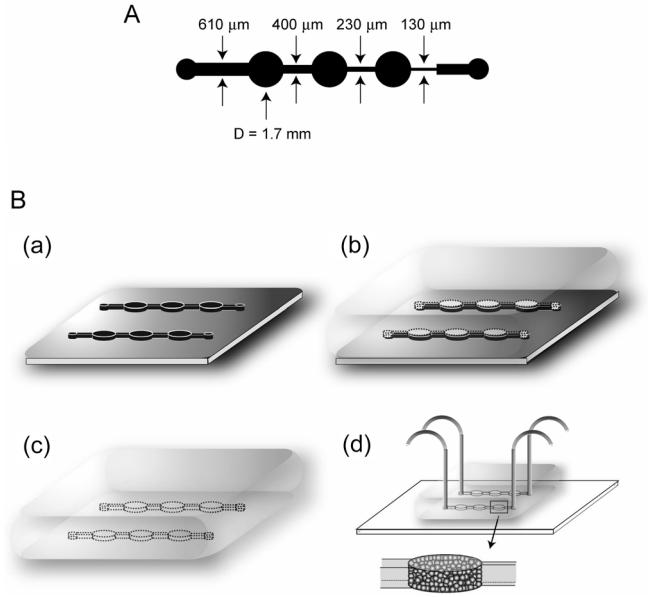

Figure 1. (A) Schematic design and dimensions of the serial microchambers and (B) overall fabrication procedures. (a) SU-8 replica master, (b) PDMS molding, (c) PDMS release from the replica master, and (d) PDMS bonding with the slide glass.

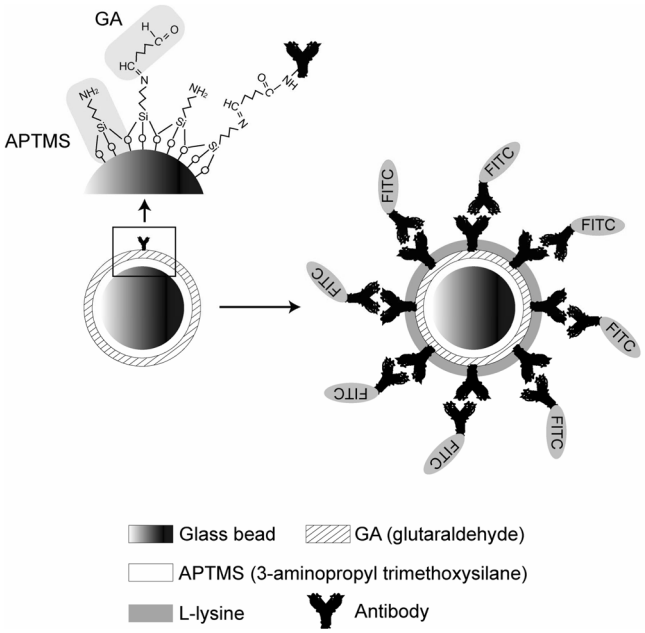

Figure 2. Surface modification of glass bead with 3-aminopropyl trimethoxysilane (APTMS) and glutaraldehyde (GA). After antibody immobilization, the rest of the surface was blocked with L-lysine followed by bonding enhancement with sodium cyanoborohydride. FITC-tagged antibody was used as a target analyte.

phosphate buffer, 2.7 mM KCl, and 137 mM NaCl. Antibody for *E. coli* O157:H7 was purchased from Kirkegaard & Perry Laboratories (KPL, Gaithersburg, MD), and *E. coli* O157:H7 43894, Salmonella enteritidis 4931, and Salmonella typhimirium 13311 were purchased from the American Type Culture Collection (ATCC).

Design of microchambers. The microfluidic device (25 mm × 10 mm) contains three microchambers connected to each other by microchannels of different widths. Figure 1A shows the design and the dimensions of the microchambers. Each microchamber is 1.7 mm and 900 μm in its diameter and height, respectively, and the volume of one microchamber is approximately 2 μL. The first microchannel which connects the inlet port to the first microchamber, is 610 μm in width; the second microchannel which connects the first to the second microchamber is 400 μm in width; the third microchannel is 230 μm in width; the fourth microchannel is 130 μm in width. The width of the fourth microchannel was designed to be smaller than the diameters of glass beads to prevent the loss of beads through the outlet port. The widths of the microchannels were designed to vary so that different-sized beads could be packed in different microchambers.

Fabrication of microchambers. PDMS microchip was fabricated by usual photolithographic method and replica molding, described elsewhere.[7] Figure 1B demonstrates the overall fabrication procedures. First, SU-8 2050 was spin-coated on a silicon wafer to create SU-8 replica master containing structures of microchambers and microchannels with approximately 900 μm in height. The surface of the SU-8 replica master was treated with the vapors of (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-1-trichlorosilane for 15 min to passivate the surface. Next, a 10 : 1 mixture of the PDMS prepolymer and curing agent was cast on SU-8 replica master to generate negative patterns of the SU-8 patterns. The PDMS prepolymer was degassed, cured at 80°C for 1 h, and peeled off. The surfaces of the PDMS replica and slide glass were treated with oxygen plasma (25 W, 30 sec) and bonded together. Holes were punched on the inlet and outlet ports and silicone tubes (ID = 1 mm, OD = 2 mm) were inserted into these ports to introduce the bead and sample solution.

Antibody immobilization on glass beads. Most of our antibody immobilization procedures follow the previously described method with slight modifications.[8] Figure 2 shows the schematic of antibody immobilization procedures. Glass beads were immersed in 10% APTMS in anhydrous toluene, sealed tightly, and reacted overnight at room temperature to form self-assembled monolayers (SAMs) of aminosilane. After removing the solution, glass beads were washed several times with toluene and acetone, and dried in an oven (60°C) for 2 h. Amino groups formed on glass beads were then reacted with 2.5% (v/v) of glutaraldehyde solution for 1 h at room temperature and washed thoroughly with distilled water. Antibody solutions were prepared in borate buffer and reacted with glutaraldehyde-treated glass beads by gently shaking overnight at 4°C. After antibody immobilization, antibody-coated glass beads were packed in microchambers using syringes. After antibody packing in microchambers, the unreacted aldehyde groups were blocked with L-lysine in borate buffer (10 mg/mL) by introducing 1.5 mL of the solution using syringe pump (KDS 220, KD Scientific, New Hope, PA, USA). To enhance antibody binding, the Schiff bases were reduced with sodium cyanoborohydride

($NaBH_3CN$) in borate buffer (20 mg/mL) by introducing 1.5 ml of the solution. After the reduction, antibody-coated glass beads were thoroughly washed with 0.5 M Tris/HCl buffer. Secondary antibody solutions (60.5 μg/mL) were prepared in PBS buffer, and introduced into the microchamber using the syringe pump.

Assays. BCA (bicinchoninic acid) assay was performed to measure the amount of antibody immobilized on the surface of glass beads. Bovine serum albumin (BSA) was used as a standard for protein concentration. Amount of immobilized antibody on the surface of glass bead was obtained by comparing the original antibody concentration in solution used for the immobilization to the remaining antibody concentration after the immobilization. The existence of amino groups on the surface of glass bead was examined by the ninhydrin test.[9,10] 5% aqueous solution of ninhydrin was prepared and aminosilane-treated glass beads were immersed in the solution for several minutes at room temperature. The existence of amino group was confirmed by the color change of glass bead into blue.

Detection. Binding of FITC-tagged anti-IgG to immobilized antibody on glass beads was evaluated by measuring the fluorescence intensity of the captured bitmap images of the microchambers using an inverted fluorescence microscope (Axioskop 2, Carl Zeiss, Germany) equipped with a high resolution CCD camera (Axio Cam, Carl Zeiss, Germany). Field emission scanning electron microscopy (FESEM) images were obtained using JSM-6700F (JEOL).

Results and Discussion

Fabrication of serial microchambers. The heights of the microchambers and microchannels were fabricated to be approximately 900 μm. Owing to relatively large height of the microstructures, glass beads were smoothly introduced into the microchambers without clogging. Glass beads stopped at the end of the third microchamber and did not pass through the outlet because the width of the microchannel (130 μm) which connects the third microchamber to the outlet was smaller than the diameter of the smallest glass bead (150 μm). By adopting this design, the microfluidic device would not need to include weir-structures which was used to pack microspheres in other microfludic devices,[11] thereby simplifying the overall fabrication procedures. Furthermore, by varying the widths of microchannels which connect individual microchambers, targeted packing of different-sized beads at desired microchambers could also be realized. With this packing strategy, the parallel assays of multiple analytes would be possible by flowing a sample solution containing multiple analytes into bead-packed microchambers containing different antibodies. In addition, relatively wide pores formed inside glass bead-packed microchambers can minimize the pressure drop and facilitate the sample flow.

Binding efficiency of antibody immobilization on glass beads. To immobilize antibody to glass beads, primary amines of antibody were covalently conjugated to carboxyl-terminated glass beads previously treated with aminosilane followed by glutaraldehyde. The existence of amino group on aminosilane-treated glass beads was confirmed by observing the color change of the glass beads into blue through ninhydrin test.

The binding efficiency of antibody immobilized on glass beads was examined by observing the fluorescence inten-

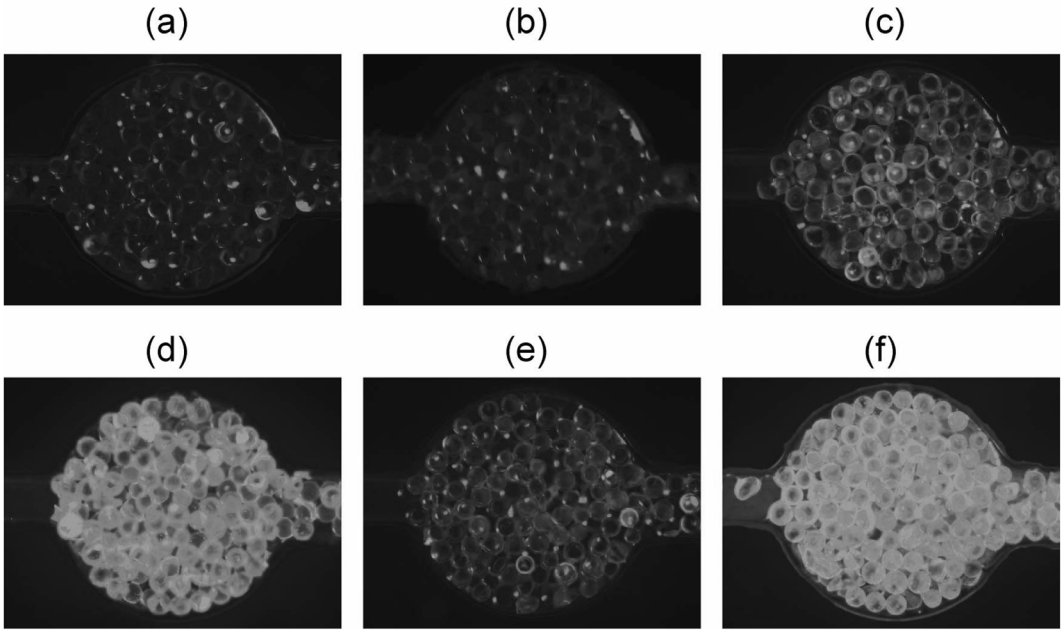

Figure 3. Fluorescence micrographs (measured at 488 nm) of (a) bare glass, (b) bare glass surface reacted with FITC-tagged anti-rabbit IgG, (c) surface-modified glass bead reacted with FITC-tagged anti-rabbit IgG but without immobilizing rabbit IgG, (d) surface-modified glass bead immobilized with rabbit IgG (500 μg/mL) and reacted with FITC-tagged anti-rabbit IgG, (e) surface-modified glass bead, immobilized with goat IgG and reacted with FITC-tagged anti-rabbit IgG, and (f) surface-modified glass bead immobilized with goat IgG and reacted with FITC-tagged anti-goat IgG.

sities from FITC-tagged anti-rabbit IgG binding to rabbit IgG previously immobilized on glass beads as shown in Figure 3. Bare glass beads did not fluoresce by themselves, and the nonspecific adsorption of FITC-tagged anti-rabbit IgG (500 μg/mL) on the surface of the untreated glass bead was negligible as represented by the absence of green fluorescence of FITC, as shown in Figures 3a and b, respectively. When FITC-tagged anti-rabbit IgG was introduced inside the microchamber previously packed with goat IgG-immobilized glass beads, FITC-tagged anti-rabbit IgG was much less bound to the glass beads, resulting in generating far less fluorescence (Fig. 3e), compared to the fluorescence obtained from the microchamber as shown in Figure 3d. Antibody binding specificities were further examined by reacting FITC-tagged anti-goat IgG to goat IgG, immobilized on glass beads (Fig. 3f). From these results, it was suggested that antibody immobilized on glass beads was not denatured over time and was sufficiently exposed to anti-IgG antibody.

Antibody immobilization on glass beads. To find out the maximum amount of antibody immobilized on glass beads, various concentrations of antibody solutions were initially prepared for antibody immobilization. Based on the BCA protein assay results, it was calculated that the average amount of goat IgG immobilized on a unit gram of glass beads were 28.4, 379, and 905 μg, respectively, when 30, 300, and 1000 μg/mL of goat IgG were used to react with glass beads. Considering that the volume of one microchamber and the density of glass are approximately 2 μL and 2.5 g/cm,[12] the amount of the glass beads packed in one microchamber was approximately 5 μg. It was further calculated that the total amounts of IgG immobilized on 5 μg of glass beads were 0.142, 1.90, and 4.53 μg, respectively. However, there seemed to be no significant difference in fluorescence intensity from two different chambers having the total amount of immobilized IgG at 0.142 and 4.53 μg, respectively, when FITC-tagged anti-goat IgG (60.5 μg/mL) was introduced into each chamber to detect immobilized antibody on the glass beads. Considering both the high cost of antibody and the sensitivity of antibody immobilized on glass beads, it was decided to use 30 μg/mL of antibody for the immobilization process on glass beads. Since the immobilization mechanism is based on covalent bonding between the amino groups of antibody and carboxyl-terminated surface of glass beads, any antibody irrespective of its animal origin could be immobilized on the surface of glass beads, as opposed to affinity-capture-based supports such as protein A or G beads in which antibody from specific animal origins can be immobilized.

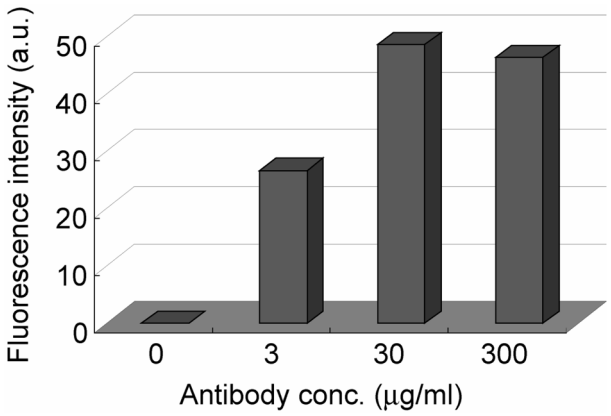

Figure 4. Fluorescence intensities of *E. coli* O157:H7 immobilized inside the microchambers when the concentrations of immobilized antibody specific for *E. coli* O157:H7 were 3, 30, and 300 μg/mL.

***E. coli* O157:H7 detection inside the microchambers.** The application potential of this microfluidic immunoassay platform was tested by detecting *E. coli* O157:H7. Antibody specific to *E. coli* O157:H7 (anti-O157 antibody) was immobilized on surface-treated glass beads at varying concentrations of 3, 30, and 300 μg/mL. 1.5 mL of diluted growth medium containing a suitable amount of *E. coli* O157:H7 expressing green fluorescence protein (GFP), equivalent to total 3.3×10^3 CFU, was later introduced into the microfluidic device packed with glass beads attaching anti-O157 antibody.

As shown in Figure 4, the maximum fluorescence of *E. coli* O157:H7 expressing GFP was observed in a microchamber in which 30 μg/mL of anti-O157 antibody was used for the antibody immobilization. The specificity of the microfluidic immunoassay platform was tested with negative

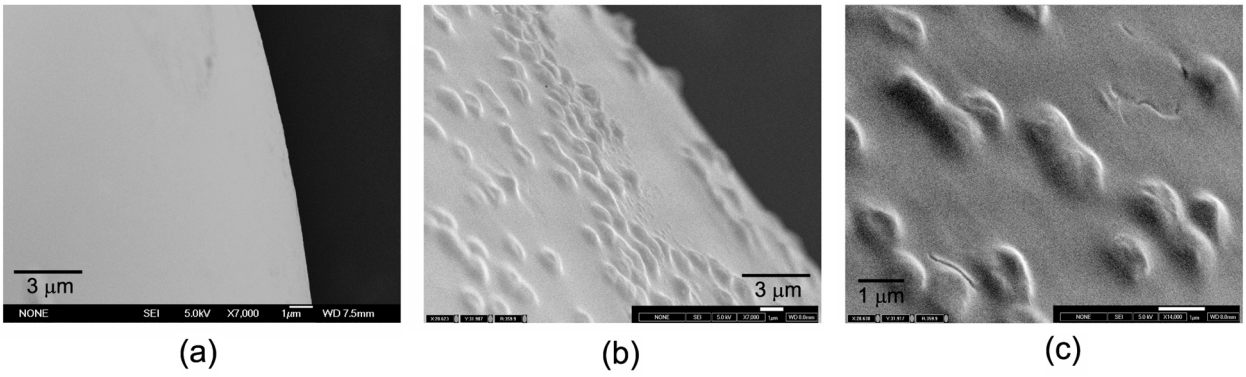

Figure 5. Scanning electron microscopy (SEM) images of (a) untreated glass beads, (b) *E. coli* O157:H7 antibody (300 μg/mL) immobilized on surface-treated glass beads, and (c) enlarged image of (b).

controls, *S. enteritidis* 4931 and *S. typhimirium* 13311 expressing GFP. No significant fluorescence was observed in microchambers with these negative controls.

The binding specificity of antibody-immobilized glass beads was further examined by observing the presence of *E. coli* O157 on the glass beads with SEM. As shown in Figures 5b and c, bacterial cells of *E. coli* O157:H7 were only observed on the surface of glass beads immobilized with O157 antibody, indicating that antibody-immobilization on glass beads has sufficient binding avidity to capture *E. coli* O157 from sample fluids flowing through the microchambers.

Summary

A solid-phase microfluidic immunoassay platform was realized in an *ad hoc* manner simply by packing antibody-immobilized glass beads in microchambers on a microfluidic device. In addition to this simplicity, the increased surface areas in microchambers offered by the bead packing allows increased amounts of antibody to be immobilized on a local area of interest, potentially enhancing the sensitivity. This platform is highly appropriate for introducing glass beads attaching several different antibodies in discrete microchambers on a single microfluidic device, thereby realizing multiple–analyte detection with a single operation. For examples, this platform can be further developed as a chamber-type microfluidic biosensor for the simultaneous detection of multiple bacterial pathogens.

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