Photochemical Immobilization of Enzyme on Inner Wall of Microchannel

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A new method for patterning several kinds of enzymes to a specific position inside the microchannel was developed for the purpose to enhance reaction efficiency of multi-channel enzyme sensor or immuno-sensor. This method was based on the photochemistry. A photoreactive crosslinker, 4-azido-2,3,5,6-tetrafluorobenzoic acid succinimidyl ester, was incubated with horseradish peroxidase (HRP). The solution was introduced into the microchannel, and then UV light irradiated the specific position of the microchannel to create the zone of HRP. After washing the microchannel, a zone of alkaline phosphatase (ALP) was similarly created in another place in the same microchannel. The activity of HRP and ALP immobilized inside the microchannel was measured using fluorogenic substrate and incident light fluorescence microscopy. The fluorescence via substrate conversion was observed only at the position where the UV light had irradiated. The result indicates that HRP and ALP are regioselectively immobilized on inner wall of microchannel with maintaining their activity. The method developed in this study has a wide range of application because two or more different functional materials can be immobilized simply to an arbitrary position inside the microchannel and will become a basic technology of micro fabrication for surface functionalization in microchemistry.

Introduction

Enzyme-Linked Immuno-Sorbent Assay (ELISA) is one of the highly sensitive determination methods for trace constituents in biological samples. The detection method is based on labeling the antigen or antibody with enzyme. A 96 holes well is generally used in ELISA as a place for the enzyme reaction. However, the technique requires a long analysis time because of the small diffusion rate of large sample molecules such as antibody or enzyme. In addition, complex operations, such as addition of reagent, washing etc., are necessary. The technique also needs large amount of samples and expensive reagents. The target sample is usually a minor constituent in living body. Thus, it is necessary to develop a new analysis method for decreasing the sample amount and the measuring time.

Recently, Micro Total Analysis System (μ-TAS) is paid attention on a global scale. [1-5] It often gives the reduction of the sample amount, the automation of analytical operation and the miniaturization of whole system. We have applied polymeric materials for microchip as substitutes of glass, which was generally used as the material of microdevices, and developed open-channel electrochromatography with low cost and easy-to-make [6-17]. It was found that the effects of size and surface were extremely important in microchemistry. In a very small space of microchannel, diffusion controlled reaction progresses extremely fast. Therefore, conducting ELISA on microchip will provide high-speed measurement, simplified operation, and reduction of sample amount.

Many researchers have reported microchip-based immunoassays system. [18-25] These were consisted of the microchip that fills gel or beads in the microchannel. We have also developed ELISA measurement using beads packed microchip. However, the method was time-consuming and had disadvantage such as clogging up of the channel, formation of particular flow path in the gel or micro-beads and so on. If the protein, such as antigen and antibody, can be immobilized on the inner wall of microchannel, such problems will be cleared up. However, to immobilize protein to a specific position on inner wall of the microchannel is very difficult because of the difficulty in making microfluidic device after the immobilization of protein, which is fragile to heat and organic solvent. One potential tool to solve the problem is the use of a photochemical reaction inside the micro channel.

In this paper, we propose the novel method for patterning protein to a specific position on inner wall of microchannel. This method is based on the photochemistry. Fig. 1 shows the principle of the photochemical immobilization. The microchannel was filled with the conjugate of crosslinker and protein, and then UV light irradiated the microchannel. Protein can be modified only to the position in which UV light was irradiated. The basic investigation of the photochemical immobilization was performed using 96 walls well, Avidin alexa fluor 488 conjugate (Avidin488) and photoreactive crosslinker, 4-azido-2,3,5,6-tetrafluorobenzoic acid succinimidyl ester

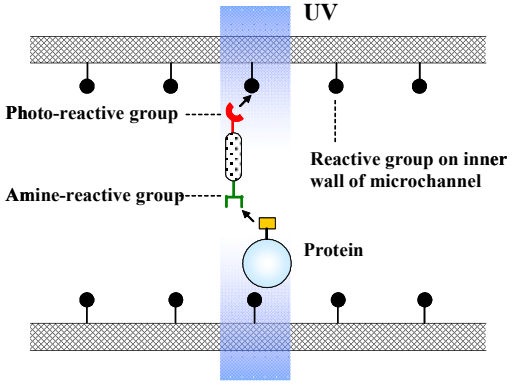

Fig. 1 Principle of photochemical immobilization

マイクロチャンネル内壁への酵素の光化学固定化

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(ATFB-SE). The technique was successfully applied to the immobilization of two different enzymes, Horseradish peroxidase (HRP) and Alkaline phosphatase (ALP), on inner wall of polystyrene or PDMS microchannel.

Experimental

Chemicals

A 96 holes well made from polystyrene (35-1172) was purchased from Becton Dickinson Co. (NJ, USA). For fabrication of master glass template, mask blank plate coated with a thin layer of 50 nm Cr/Cr_2O_3 (DUFR-2506 p-L) was received from ULVAC equipment sales Inc. (Tokyo, Japan). Positive type photoresist (PMER P-RZ300) and developer solution (PMER P-1S) were obtained from Tokyo Ohka Kogyo (Kawasaki, Japan). For chip fabrication, polystyrene plate was from Tamiya Inc. (Shizuoka, Japan). PDMS micro-fluidic devices were fabricated by curing the prepolymer components of Sylgard 184 (Dow Corning, MI, USA) on a template. Avidin488, ATFB-SE, HRP, Amplex Red and fluorescein diphosphate (FDP) were purchased from Molecular Probes (Eugene, OR, USA). ALP was obtained from ELASTIN PRODUCTS (Owensville, Missouri, USA). Other chemicals used were of analytical reagent grade. Water was purified using a Milli-Q system (Nihon Millipore, Tokyo, Japan). All buffer solutions were filtered through a JHWP04700 membrane filter (0.45 μm, Nihon Millipore, Tokyo, Japan) and degassed with a vacuum pump before use.

Fabrication of master glass template

The preparation of master glass template was performed through photolithography and wet-chemical etching procedure followed by replication. Details of the procedure were described in previous paper. [9] Briefly, a mask blank plate was coated with thin layer of positive photo resist PMER P-RZ300 film by a spin coater (K-359 S-1, Kyowa Riken, Japan) at 600 rpm for 2 min and baked in an oven at 85 ºC for another 15 min. A serpentine channel design (made by Illustrator 10 and printed out on a positive film by Kodak Co. Ltd., Japan) was transferred onto the mask plate by exposing to a long wavelength ultraviolet lamp (FLB-15, Toshiba, Tokyo, Japan) for about 2 min, followed by developing in PMER P-1S solution for about 3 min. The template with channel design was further baked at 85 ºC for 15 min. The Cr / Cr_2O_3 layer was then removed with the mixture of 17% (w/v) cerium (IV) diammonium nitrate and 5% (v/v) perchloric acid solution to define the channel on the mask plate. After rinsing in 2 M HNO_3 for 5 min, a positive relief channel was obtained by etching the glass in 1M NH_4F / 1M HF solution at room temperature for 50 min. The microchannel consisted of straight line with 4 cm long, 1 mm wide and 50 μm deep. The master glass was kept in a clean box before use.

Apparatus

Microscopic fluorescence imaging system used was described in previous paper. [26-30] Briefly, the system consisted of an inverted fluorescence microscope (IX71, OLYMPUS, Tokyo, Japan), filter set of excitation filter, dichroic mirror and emission filter (XF100-2 or XF102-2, Omega optics Inc. VT, USA) and CCD camera (RETIGA1300, QImaging, BC, Canada). Fluorescence images were recorded by capture software (QCAPTURE, QImaging, BC, Canada) on PC.

Microscopic fluorescence analysis system consisted of a He/Ne laser (543 nm, 5 Mw, 05-LGP-173, MELLES GRIOT, CA, USA), an incident-light fluorescence microscope (BX51, OLYMPUS, Tokyo, Japan), filter set of excitation filter, dichroic mirror and emission filter (XF100-2 or XF102-2, Omega optics Inc. VT, USA), a photomultiplier tube (R535, Hamamatsu Photonics, Shizuoka, Japan), a laboratory-made amplifier, a chromatographic data processing system (CR-7A, Shimadzu, Kyoto, Japan) and a syringe pump (PHD2000, HARVARD Apparatus, USA).

Results and discussion

Immobilization of avidin on the wall of 96 holes well

Avidin alexa fluor 488 conjugate (Avidin488) was immobilized on the wall of polystyrene 96 holes well. At first, 50 μL of 1 mg / mL ATFB-SE in nitromethane was added to the well and then the well was dried at 60℃ for 30 min. After 10 min illumination of ultraviolet light from sterilization lamp (GL-6, Toshiba, Tokyo, Japan), the well was rinsed with copious amounts of nitromethane and then dried. Next, 50 μl of 1 mg / mL Avidin488 in 0.1 M carbonate buffer (pH 8.3) was added to the well. The well was left at room temperature for 1 hour and then rinsed with the buffer and water.

Fig. 2 shows the fluorescent image of the 96 holes well on which Avidin488 was immobilized. A strong fluorescence was observed from the well which UV light irradiated. On the other hand, only weak background fluorescence was observed from other well. This shows that Avidin488 was immobilized on polystyrene plate through ATFB by UV irradiation. Fluorescence did not weaken though the well is washed by 6M guanidine and SDS solution. This means that the immobilized Avidin488 did not flow out by the washing with the solution which is high ionic strength and contain surfactant because the immobilization was based on the formation of covalent bonding with the wall

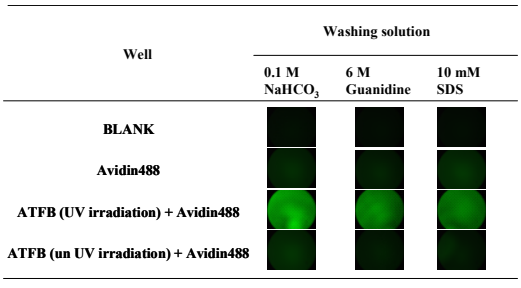

Fig. 2 Fluorescent image of the 96 holes well

Immobilization of enzyme on inner wall of polystyrene microchannel

We studied the immobilization of enzyme on inner wall of polystyrene microchanne Fabrication process of polystyrene microchip is as follows. The master glass template was placed on a polystyrene plate with 1.7 mm thick and then the microchannel was thermally transcribed

on the polystyrene plate. The both ends of microchannel on the plate were punched for sample installation and exhaust. The plate was thermally bonded with a flat polystyrene plate of 0.2 mm thick and then two PEEK tubes were mounted to the device to inject or exhaust a solution.

1 mL of 10 mg/mL enzyme in 0.1 M carbonate buffer (pH 8.3) and 100 μL of 10 mg/mL ATFB-SE in DMSO was mixted. After incubation for 1 hour at room temperature, 100 μL of 1.5 M Hydroxylammonium chloride was added to the solution to quench the amine-reactive group. After incubation for 1 hour at room temperature, the solution was preserved at -20 ℃.

The solution containing the conjugate of ATFB-SE and HRP was introduced into the polystyrene microchannel, and then the beam from He-Cd laser (325 nm, 200 mW) irradiated the specific position of the microchannel to create the zone of HRP. After washing the microchannel, the zone of ALP was similarly created in another place in the same microchannel.

The activity of HRP and ALP immobilized on the inner wall of polystyrene microchannel was investigated using Amplex Red and FDP. Amplex Red is converted into Resorufin, which is highly fluorescent, by HRP in the presence of hydrogen peroxide. FDP is dephosphorylated by ALP to give highly fluorescent Fluorescein. The microchannel was filled with the solution containing Amplex Red and hydrogen peroxide or FDP. The resulting pattern of immobilized enzymes was imaged under an inverted fluorescence microscope with a CCD camera. Fig. 3 shows the fluorescence images of HRP and ALP immobilized on the inner wall of polystyrene microchannel. Fluorescence via substrate conversion was observed only at the position where the beam from He-Cd laser had irradiated. The misting of the fluorescent images in surroundings is probably due to the diffusion of resulting molecules, resorufin and fluorescein. This indicates that HRP and ALP are regioselectively immobilized on inner wall of microchannel with maintaining their activity.

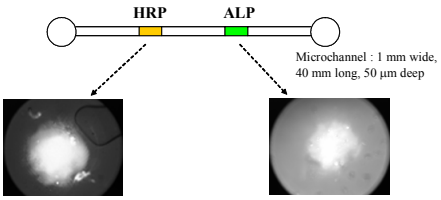

Fig. 3 Fluorescence images of HRP and ALP immobilized on the inner wall of polystyrene microchannel.

Immobilization of enzyme on inner wall of PDMS microchannel

Fabrication of polystyrene microchip is very difficult. Thus, we also studied the immobilization of HRP inside the PDMS microchannel. Fabrication process of PDMS microchip is as follows. Two Teflon tubes were placed on the grass template using water-soluble glue for sample installation and exhaust. 10 g of PDMS base was mixed with 1 g of curing agent. The mixing solution was degassed with a vacuum pump and then 4 g of the mixture was poured onto the glass template with positive relief channel to duplicate the channel, while 4 g of the was used to make a cover plate with four reservoirs. After curing at 60 ˚C for 1 h, the two PDMS molds were peeled away and then exposed to plasma in a Sanyu Electron (Tokyo, Japan) Quick Coater SC-701 (etching mode, 3 mA for 30 s). The two pieces were immediately brought together and the mold was placed in an oven at 120 ˚C for 4 h to bond irreversibly. Then, two PEEK tubes were mounted to the device to inject or exhaust a solution.

Fabrication of PDMS microchip is easy, but PDMS has strong non-specific adsorption surface for protein. Thus, after blocking with BSA, HRP was immobilized on inner wall of microchannel. Fig. 4 shows the cross sectional view of the microchip. Fluorescence is not observed from the position that HRP was not immobilized, while strong fluorescence was observed from the HRP immobilized position.

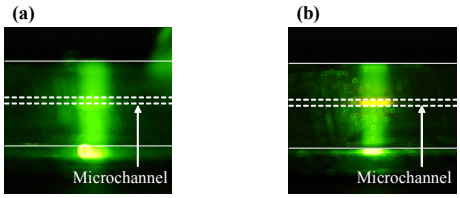

Fig .4 Cross sectional view of the microchip.

(a) Position that HRP was not immobilized.

(b) HRP immobilized position.

Fluorescence intensity was measured at the different position along the micro channel. The substrate solution (0.1 M phosphate buffer (pH 7.4) containing 0.1 mM Amplex Red and 2mM hydrogen peroxide) kept introduced into the microchannel during the measurement at 10 mL/min. Fig. 5 shows the variation of fluorescence intensity in the PDMS microchannel. Fluorescence intensity from the UV irradiated microchannel jumped dramatically after the HRP immobilized position. This means that HRP is regioselectively immobilized on inner wall of microchannel with maintaining their activity.

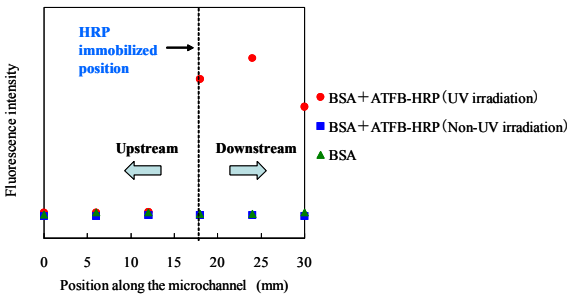

Fig. 5 Variation of Fluorescence Intensity in the PDMS Microchannel

Microchannel : 300 μm wide, 40 mm long, 20 μm deep

Flow rate : 10 μL/min

Conclusion

A new method for patterning protein on inner wall of microchannel was developed for the purpose to enhance reaction efficiency of multi-channel enzyme sensor or immuno-sensor. Two different enzymes, HRP and ALP, were successfully immobilized on inner wall of

microchannel with maintaining their specific activities. The method developed in this study has a wide range of application because two or more different functional materials can be immobilized simply to an arbitrary position inside the microchannel. The technique will become a basic technology of micro fabrication for surface functionalization in microchemistry.

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