# Photochemical Immobilization of Protein on Inner Wall of Microchannel

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(Received November 29, 2004; CL-041444)

A novel strategy for patterning several kinds of proteins in a specific position of a microchannel is presented. Horseradish peroxidase (HRP) and alkaline phosphatase (ALP) are covalently immobilized on a polystyrene microchannel surface through a photoreactive crosslinker with an azide group at one end and a succinimide group at the other by UV irradiation. The activities of HRP and ALP are evaluated using Amplex Red and Fluorescein diphosphate, respectively. The fluorescence has been observed only at the enzyme-immobilized position.

Enzyme-linked immuno-sorbent assay (ELISA) is one of the most highly sensitive analytical techniques for the determination of trace constituents in living body and is commonly used in the field of clinical inspection. A 96-well microtiter plate is often used as a place for the antigen-antibody and enzyme reactions. However, the complex operations, such as the addition of reagents and the washing of the well, are necessary. A long analysis time is also required because of the small diffusion rate of the proteins such as antigen and antibody.

Recently, micro total analysis system ($\mu$-TAS)[1–5] is paid attention on a global scale. $\mu$-TAS has the ability to perform a series of analytical operation on a microchip and often gives the reduction of reagent and the automation of analytical operation as well as the miniaturization of entire system. A very small space of microchannel accelerates the diffusion controlled reaction.[5] An accomplishment of ELISA in the microchannel therefore leads to a decrease in the analysis time.

The ELISA methods using the gel or micro-beads packed microchip have been reported.[6–8] However, these methods were time-consuming and had some disadvantages, such as the clogging up of the microchannel, the formation of particular flow path in the gel or micro-beads and so on. If proteins, such as antigens and antibodies, can be immobilized on the inner wall of microchannel, such the problems will be solved. Enzyme-immobilized microchannels have been developed.[9–11] However, the immobilization of several kinds of proteins in the specific position on inner wall of the microchannel is very difficult because of the difficulty in producing micro-fluidic devices after the immobilization of proteins which are usually fragile to heat and organic solvent. One potential tool to solve the problems is the use of a photochemical reaction inside the micro channel.[12,13]

Herein, we describe a novel method based on photochemistry for patterning several kinds of protein in the specific position on the inner wall of microchannel. Avidin alexa fluor 488 conjugate (Avidin-488) was first immobilized on the wall of a polystyrene 96-well microtiter plate through a photochemical crosslinker, 4-azido-2,3,5,6-tetrafluorobenzoic acid succinimidyl ester (ATFB-SE), by the irradiation of UV light. The method was successfully applied to the regioselective immobilization of two kinds of enzymes, horseradish peroxidase (HRP) and alkaline phosphatase (ALP), in the specific position on the inner wall of a polystyrene microchannel.

A model 35-1172 polystyrene 96-well microtiter plate was purchased from Becton Dickinson Co. (NJ, USA). For the chip fabrication, a polystyrene plate was obtained from Tamiya Inc. (Shizuoka, Japan). Avidin-488, 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). 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.

The microscopic fluorescence imaging system used was described in the previous paper.[13] Briefly, the system consisted of an inverted incident light fluorescence microscope (IX71, OLYMPUS, Tokyo, Japan), the filter set of an excitation filter, a dichroic mirror and a emission filter (XF100-2 or XF102-2, Omega optics Inc. VT, USA) and a CCD camera (RETIGA1300, QImaging, BC, Canada). Fluorescence images were recorded by capture software (QCAPTURE, QImaging, BC, Canada) on a PC.

Avidin-488 was immobilized on the wall of the polystyrene 96-well microtiter plate by the procedure below. 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 °C for 30 min. After the illumination of UV light from UV lamp (254 nm, GL-6, Toshiba, Tokyo, Japan) for 10 min, the well was rinsed with copious amounts of nitromethane and then dried. 50 μL of 1 mg/mL Avidin-488 in 0.1 M carbonate buffer (pH 8.3) was added to the well. The well was left at room temperature for 1 h and then rinsed with the buffer and water.

Figure 1 shows the fluorescence intensity of the wells after washing with 0.1 M carbonate buffer (pH 8.3), the buffer containing 6 M guanidine, and the buffer containing 10 mM SDS. As can be seen from the figure, washing the well with the buffer containing 6 M guanidine or 10 mM SDS has decreased a non-specific adsorption of Avidin-488. A strong fluorescence signal from the well which UV light irradiated indicates that Avidin-488 is bound to the carbon atom on the surface of polystyrene well through ATFB-SE. The standard deviations obtained on the well photochemically immobilized were higher than that obtained on the other wells. The problems, such as the non-specific adsorption of proteins and the reproducibility, could be overcome by using a protein blocking solution and optimizing the irradiation time of UV light, the composition of buffer, the concentration of protein, and so on.

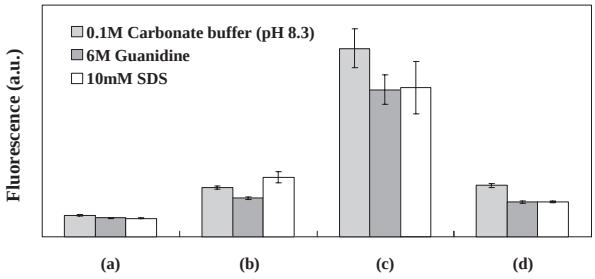


**Figure 1.** Fluorescence intensity of the polystyrene 96-well microtiter plate after washing with 0.1 M carbonate buffer (pH 8.3), the buffer containing 6 M guanidine, and the buffer containing 10 mM SDS. (a) blank well, (b) Avidin-488-adsorbed well, (c) Avidin-488-immobilized well through ATFB-SE (UV irradiation), and (d) Avidin-488-immobilized well through ATFB-SE (no UV irradiation).

The immobilization method in order to create each zones of several kinds of enzymes on the inner wall of the polystyrene microchannel was investigated. The fabrication process of the polystyrene microchip is as follows. The preparation of the convex master glass template has been described in detail elsewhere.[14] The master glass template with the microchannel (40-mm long, 1-mm wide and 50-μm deep) 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 the 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 in order to inject or exhaust a solution.

The conjugate of ATFB-SE and enzyme was prepared beforehand as follows. 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 were mixed. After the incubation for 1 h at room temperature, 100 μL of 1.5 M hydroxylammonium chloride was added to the solution in order to quench the amine-reactive group of ATFB-SE. After the incubation for 1 h at room temperature, the solution was preserved at −20 °C.

The zones of two enzymes, HRP and ALP, were created at the separate position of the polystyrene microchannel. The solution containing the conjugate of ATFB-SE and HRP was introduced into the microchannel, and then the beam from He–Cd laser (325 nm, 200 mW, KIMMON electric Co., Ltd., Tokyo, Japan) irradiated the specific position of the microchannel in order to create the zone of HRP. After washing the microchannel, the zone of ALP was also created at another position of the microchannel in the same manner.

The activities of HRP and ALP immobilized on the inner wall of the polystyrene microchannel were investigated using Amplex Red and FDP, respectively. 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 0.1 mM Amplex Red and 2 mM hydrogen peroxide or 10 μM FDP. The resulting pattern of the enzymes immobilized was imaged under the inverted fluorescence microscope with a CCD camera.

Figure 2 shows the fluorescence images of HRP and ALP immobilized on the inner wall of the polystyrene microchannel.

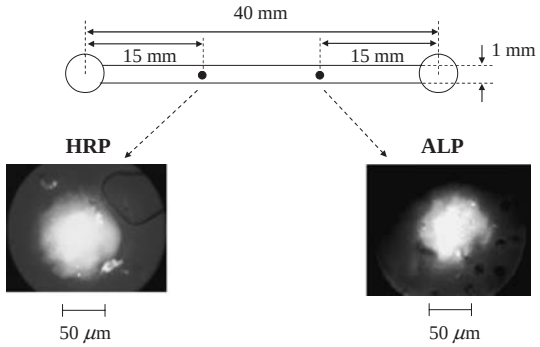


**Figure 2.** Fluorescence images of the HRP and ALP immobilized on the inner wall of the polystyrene microchannel. The depth of the microchannel is 50 μm.

The fluorescence was observed only at the position where the beam from He–Cd laser had irradiated. This indicates that HRP and ALP are regioselectively immobilized on the inner wall of the microchannel with maintaining their activity. The misting of the fluorescence images in surroundings is probably due to the diffusion of the resulting molecules, i.e., resorufin and fluorescein. Although the diameter of the beam irradiated was ca. 1 mm, the diameter of the fluorescence observed was only ca. 70 μm. This is because the laser power is the strongest in the center and weakens in the periphery.

We have developed a novel strategy for photochemical immobilization of protein on inner wall of microchannel. The technique has a wide range of application because two or more different functional proteins can be immobilized simply in an arbitrary position of the microchannel. The technique will become a basic technology of micro fabrication for surface functionalization in microchemistry.

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Published on the web (Advance View) February 12, 2005; DOI 10.1246/cl.2005.358