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Regioselective Immobilization of Protein on the Surface of Polymeric Microchannel by Photo-reactive Crosslinker

Hizuru Nakajima, Toru Fukuda, Miki Takizawa, Takuya Shimosaka, Toshiyuki Hobo and Katsumi Uchiyama*

Department of Applied Chemistry, Graduate School of Engineering, Tokyo Metropolitan University

1-1 Minamiohsawa, Hachioji, Tokyo 192-0397, Japan

Fax: 81-426-77-2821, e-mail: uchiyama-katsumi@c.metro-u.ac.jp

We proposed in this paper a novel immobilization method of functional protein on the inner surface of microchannel chip. A photochemical crosslinker with an azide group at one end and succinimide group at the other was first patterned on a polymeric microchannel by UV irradiation, and then avidin was bound to the crosslinker on the surface through the amine reactive group. Non-specific binding of protein, which is usually a large bar to the sensitive and specific measurement, could be removed using 6 M guanidine solution. The activity of chemically-bonded avidin was still remained after the washing. Biotinylated protein was regioselectively bonded to the avidin-patterned microchannel. We could successfully develop a novel method for photo-chemical patterming of protein on the inner surface of micro channel chip with avidin-linked patterning process.

Key words: Immobilization, Protein, Microchip, Modification, Photo-reactive crosslinker

1. INTRODUCTION

Enzyme-Linked Immuno-Sorbent Assay (ELISA) is one of the most powerful determination methods of trace constituents in living body and is widely used in the field of clinical analysis. A 96 holes well is generally used in ELISA as a place for the enzyme reaction, and the samples can be measured with high sensitivity by labeling the antigen or the antibody with enzyme. However, the technique requires a long analysis time because of the small diffusion rate of large sample molecules such as antibody or enzyme. The technique also needs a large amount of samples and reagents, and the target sample is usually a minor constituent included in blood and body fluid from a living body. Thus the decrease of the sample amount and measuring time for ELISA measurement is strongly needed.

Miniaturized Total Chemical Analysis System (μ-TAS), where a series of chemical analysis operation such as a preprocessing, extraction, reaction, separation and the detection of the sample that has been executed at the laboratory-level up to now is executed on the chip of several-centimeter corner; so called microchip, is paid great attention in recent years [1-9]. We have also proposed a use of the polymeric microchip including simple surface modification method and also developed an easy fabrication process for them [10-19]. In μ-TAS, the flow channel of 10-200 μm in width and 1-50 μm in depth made on the glass or polymeric substrates is used as a place for reaction and separation. The diffusion time of materials in the very small microchannel is extremely short. Mean diffusion time of a molecule is proportional to the square of the size. Thus it is thought that conducting antigen-antibody reaction, which is principle of ELISA in microchip, provides the saving of sample amount, the high-speed measurement and the miniaturization of entire device. Actually, there are a lot of examples of immobilizing the enzyme in the microchannel. However, there are few studies on the improvement of separation selectivity and detectability by immobilizing several kinds of enzymes at their specific positions.

The purpose in this study is to develop a base of the functional proteins at the specific position inside the microchannel and to propose a compact device that can execute ELISA used in wide fields of biochemical analysis, medical diagnosis, and environmental analysis with high accuracy and speed. This paper describes a new region-selective immobilization method of enzyme on the microchannel inner wall by introducing amine reactive group into the particular position of micro channel surface using photochemical reaction.

2. EXPERIMENTAL SECTION

2.1 Chemicals and Materials

All reagents used in this study were of analytical reagent grade unless otherwise stated. Sodium dihydrogenphosphate, disodium hydrogenphosphate, sodium hydrogen carbonate, and nitromethane were obtained from Kanto Chemicals (Tokyo, Japan). Guanidine hydrochloride salt, 30% hydrogen peroxide, and pH standard solutions were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hydroxylammonium chloride and dimetyl sulfoxide (DMSO) were obtained from Aldrich (Milwaukee, WI, USA) and NAKALAI TESQUE (Kyoto, Japan) respectively. 4-azido-2,3,5,6-tetrafluorobenzoic acid succinimidyl ester (ATFB-SE), ELISA grade horseradish peroxidase (HRP), 10-acetyl-3,7-dihydroxy phenoxazine (Amplex Red), avidin alexa fluor 488 conjugate (Avidin488), avidin-chicken egg white (Avidin), and fluorescein diphosphate tetraammonium salt (FDP) were purchased from Molecular Probes

(Eugene, OR, USA). Alkaline phosphatase chicken intestine (ALP) and biotinylamidocaproic acid-N-hydroxysuccinimide ester (Biotin-X-NHS) were obtained from ELASTIN PRODUCTS (Owensville, Missouri, USA) and RESEARCH ORGANICS (Cleveland, OH, USA) respectively. Bovine serum albumin (BSA) was purchased from CALBIOCHEM (San Diego, CA, USA). Aqueous solutions were prepared with water purified by a Nihon Millipore (Tokyo, Japan) Milli-Q system. Phosphate buffer was prepared from NaH_2PO_4 and Na_2HPO_4. Carbonate buffer was prepared from $NaHCO_3$. All buffers were filtered through a 0.45 μm membrane filter before use. A Tamiya (Shizuoka, Japan) transparent plastic plate (0.4 or 1.7 mm thickness) for model was used as a polystyrene plate.

2.2 Apparatus

The microscopic fluorescence analysis system used for the confirmation of immobilized HRP and ALP is shown in Fig. 1. Nd/YAG laser (500 mW, GCL-050-M, CrystaLaser, Reno, NV, USA) and Ar^+ laser (100 mW, LGK-7872-M, LASOS, Jena, Germany) were used to generate an excitation light of 532 nm and 488 nm respectively. The beam was led to incident light fluorescence microscopy (BX40, OLYMPUS, Tokyo, Japan), and then reflected by a mirror onto the part of immobilization on polystyrene plate. The fluorescence was collected by an objective lens and then filter thought a band pass filter. Basic fluorescence system used was incident light fluorescence microscope setup (OLYMPUS, Tokyo, Japan). Filter set used was U-MW1G2 for HRP and U-MW1B2 for ALP. The fluorescence was transformed into an electric signal by a photomultiplier tube (R535, Hamamatsu Photonics, Shizuoka, Japan). The electric signal was amplified by a laboratory-made amplifier and recorded by chromatographic data processing system (CAC, Nihon Filcon, Tokyo, Japan).

For the confirmation of immobilization of Avidin488, the inverted microscopic fluorescence analysis system used is shown in Fig. 2. A light from mercury lamp was focused by a lens (f=200) in microscopy (IX71, OLYMPUS, Tokyo, Japan), passed through the band-pass filter (U-MW1B2), and then reflected by a mirror onto the part of immobilization on polystyrene plate. The fluorescence images were recorded by CCD camera (RETIGA1300, QImaging, BC, Canada) and capture software (QCAPTURE, QImaging, BC, Canada) on PC. The exposure time was green; 30 s, red; 40 μs, blue; 40 μs.

Micro reactor experiment was performed using a Furue science (Tokyo, Japan) model JP-V micro feeder accommodated with a syringe with a 10 ml capacity and a Rheodyne (Cotati, CA, USA) Model 7125 sampling valve equipped with a 5 μl loop.

2.3 Procedure

The procedure of immobilization of HRP and Avidin488 on the polystyrene plate is as follows. A polystyrene plate (0.4 mm thickness) was cleaned by soaking in 6M-guanidine hydrochloride. To the plate rinsed with water and nitromethane, 10 μl of 1 % (w/v) ATFB-SE in nitromethane was dropped and then the

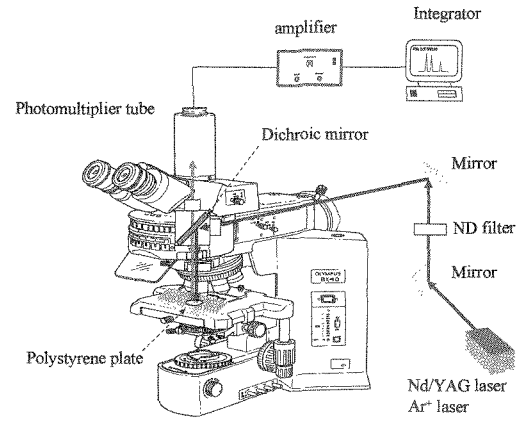

Fig. 1 Schematic diagram of microscopic fluorescence analysis system used for confirmation of immobilization of HRP and ALP.

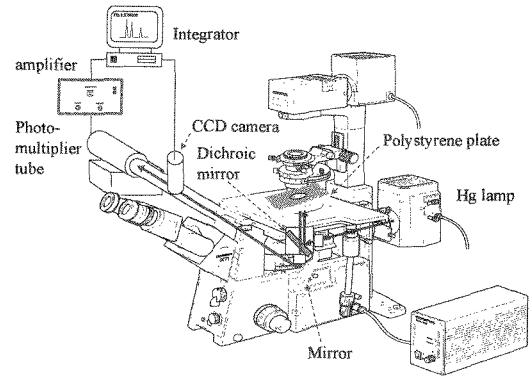

Fig. 2 Schematic diagram of microscopic fluorescence imaging system used for confirmation of immobilization of Avidin488.

plate was dried at 60℃ for 30 min. After 10 min illumination of ultraviolet light, the plate was rinsed with copious amounts of nitromethane. Onto the part of immobilization of ATFB-SE, 10 μl of 1 % (w/v) HRP in 0.1 M phosphate buffer (pH 7.0) or Avidin488 in 0.1 M carbonate buffer (pH 8.3) was dropped. The plate was left at room temperature for 2 hours and then rinsed with the buffer and water.

The immobilization process of Avidin488 or Avidin on the surface of polystyrene microchannel was as follows. ATFB-SE was dissolved in nitromethane to the concentration of 0.5 % (w/v). The solution was dropped onto the polystyrene cover plate (1.7 mm thickness), with two holes for introduction of solutions, and spin-coated by means of a spin-coater. A photo-mask plate that two holes (1cm corner) were punched was put on the plate, and ultraviolet light was irradiated for 10 min. After washing with copious amounts of nitromethane, the plate was connected to a channel plate by thermal bonding method. The channel plate was made beforehand by means of hot-embossing with the convex glass template that prepared through a photolithographic and a wet-chemical etching procedure. The microchannel used in this study was a straight line of 60 μm in depth, 1 mm in width, and 4 cm in length. The microchannel of the chip fabricated was rinsed with 6M-guanidine hydrochloride, filled with 0.5 % (w/v) Avidin in 0.1 M carbonate buffer (pH 8.3) for 2 hours and then rinsed with the buffer and water.

Biotinylated ALP was conflated as follows. 10 μl of 1 % (w/v) Biotin-X-NHS in DMSO was mixed with 200 μl of 1 % (w/v) ALP in 0.1 M carbonate buffer (pH 8.3) for 1 hour. To the solution, 20 μl of 1.5M hydroxylammonium chloride in 0.1 M carbonate buffer (pH 8.3) was added as stop reagent, and then the solution was mixed for 1 hour.

The immobilization of ALP on the surface of microchannel was performed as follows. The Avidin immobilized microchannel was rinsed with 6M-guanidine hydrochloride, filled with 1 % (w/v) BSA in 0.1 M phosphate buffer (pH 8.3) for 1 hour, and then rinsed with the buffer. The biotinylated ALP solution was introduced into the microchanel for 1 hour, and then the channel was rinsed with phosphate buffer.

3. RESULTS AND DISCUSSION

3.1 Immobilization of HRP on polystyrene plate

HRP was immobilized on the surface of the polystyrene plate. As a means of the immobilization of HRP, ATFB-SE as photo-reactive crosslinker was used. Fig. 3 shows the photo-reactive crosslinking of protein. ATFB-SE having an azido group in the structure bonds with the polystyrene by the UV irradiation. In addition, ATFB-SE has a succinimidyl ester group so that it reacts with HRP having an amino group in the end. As a result, HRP is immobilized on the surface of the polystyrene board through ATFB-SE.

The state of the immobilization of HRP was evaluated by using Amplex Red that reacted in the peculiarity with HRP. Amplex Red is hydrolyzed by HRP and generates Resorufin that is the phosphor. Therefore, a reactive amount of HRP, that is, the amount and revitalization of HRP can be examined by measuring the fluorescence intensity of Resorufin. 10 μl of 0.1 mM Amplex Red - 2 mM hydrogen peroxide in 0.1 M phosphate buffer (pH 7.0) was dropped and then the fluorescence intensity was determined by means to irradiate the laser for five seconds at intervals of one minute. Fig. 4 shows the time course of the fluorescence intensity of Resorufin by the HRP-catalyzed oxygenation of Amplex Red. The changes in the fluorescence intensity in the spot to which HRP was not dropped (Blank, the spot to which only ATFB-SE was dropped, and the spot to which ATFB-SE and hydroxylammonium chloride was dropped) were very small. The fluorescence intensity in the blank and the spot to which only ATFB-SE was dropped increased slightly after 20 minutes because Amplex Red was slightly hydrolyzed without the existence of HRP. On the other hand, the fluorescence intensity in the spot to which ATFB-SE and HRP were dropped increased with increase of the time and almost became constant after 18 minutes. This shows that HRP is immobilized on the surface of polystyrene through ATFB-SE. The fluorescence intensity in the spot to which only HRP was dropped also increased. This is probably due to the non-specific adsorption of HRP to the polystyrene. In the spot to which 0.1M hydroxylamine hydrochloride was dropped, the succinimidyl ester group of ATFB-SE should have been lost. Therefore, the increase in the fluorescence intensity in the spot to which ATFB-SE, the hydroxylamine hydrochloride and HRP are dropped is due to non-specific adsorption. The fluorescence intensity was smaller than that in the spot to which only HRP is dropped and thus it is supposed that ATFB-SE suppresses non-specific adsorption of HRP.

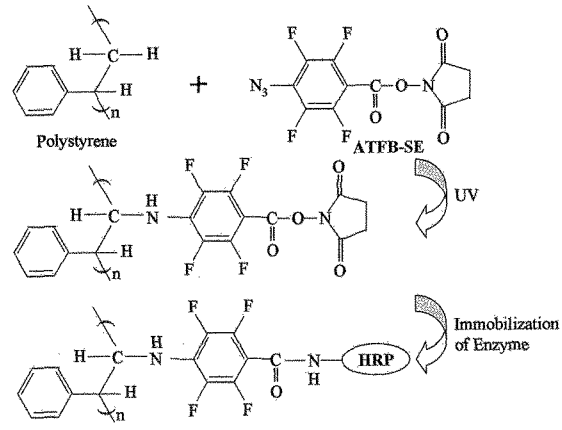

Fig. 3 Photo-reactive crosslinking of protein.

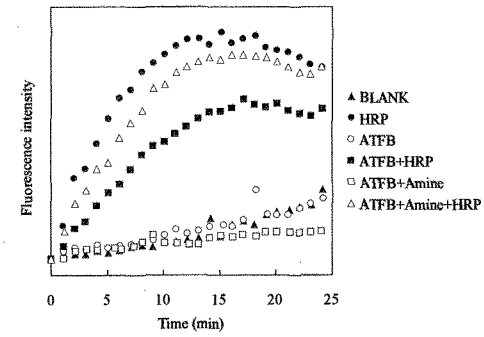

Fig. 4 Time course of the fluorescence intensity of Resorufin by the HRP-catalyzed oxygenation of Amplex Red.

3.2 Immobilization of Avidin488 on polystyrene plate

The physically adsorbed HRP cannot be removed without any loss of its activity by washing with guanidine hydrochloride salt solution because the stability of HRP is very low. Therefore, a similar examination was performed by using Avidin, which is very stable against heat shock, Guanidine, pH change and Trypsin. The Avidin used in this study was fluorescence-labeled Avidin with Alexa fluor 488 and thus fluorescence intensity can be measured without a complex operation like 3.1, and the immobilization of protein can be confirmed. The fluorescence images of the same region as in Fig. 4 were taken with the device shown in Fig. 2. The exposure time was green; 30 s, red; 40 μs, and blue; 40 μs. The coating method of ATFB-SE and the cleaning efficiency in the guanidine hydrochloride salt solution to physical adsorption of protein were examined. Fig. 5 shows microphotographs of the part of immobilization of Avidin488 on polystyrene plate. The spot to which ATFB-SE and Avidin488 are dropped showed strong fluorescence, while the fluorescence was hardly observed in BLANK. This shows that Avidin488 was immobilized on polystyrene plate through ATFB. Although the fluorescence was also observed in the spot to which only Avidin488 was dropped, after washing with 6M guanidine hydrochloride salt solution, the fluorescence was hardly observed, while the binding activity of Avidin was still remained. The results show that the

non-specific adsorption of protein could be removed by cleaning the polystyrene plate with 6M guanidine hydrochloride salt solution without the loss of the binding activity of Avidin. When ATFB-SE was coated thickly, the irregularity of the immobilization of Avidin488 was larger than that when ATFB-SE was spin-coated. This is probably due to the differences of the intensity of ultraviolet light at the surface of the polystyrene surface.

3.3 On-line regioselective immobilization of ALP on the inner surface of polystyrene microchannel

It was found that Avidin488 could be immobilized to the specific position on the inner surface of polystyrene microchannel by introduction of ATFB-SE to the position using photo-mask plate. We applied this result to the on-line regioselective immobilization of enzyme on the inner wall of polystyrene microchannel. Biotinylated ALP was bound to the immobilized Avidin. The enzymatic activity of the immobilized ALP was evaluated by using FDP that was hydrolyzed with ALP to give fluorescein. 0.1 M phosphate buffer (pH 8.3) was used as the mobile phase, and 5 μl of 1 x 10^{-5} M FDP dissolved in the buffer was introduced into the microchannel via sampling valve. The flow rate of the mobile phase was regulated by micro feeder, and the fluorescence was detected after the part of immobilization of ALP. Fig. 6 shows plots of fluorescence intensity versus flow rate. The fluorescence intensity was shown by peak height. Fluorescence by ALP combined with Avidin was larger than that after cleaning with 6 M guanidine hydrochloride salt solution in each flow rate. The result means that ALP was immobilized by ATFB-Avidin and Biotin. The fluorescence intensity was increased with decrease of the flow rate. It was found that the reaction time became long with decrease of the flow rate, and thus the reaction efficiency increased.

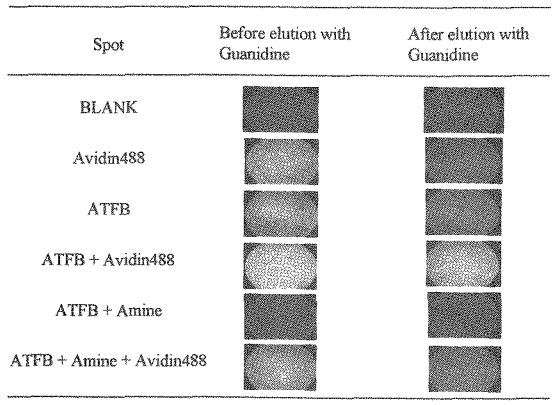

Fig. 5 Microphotographs of polystyrene plate.

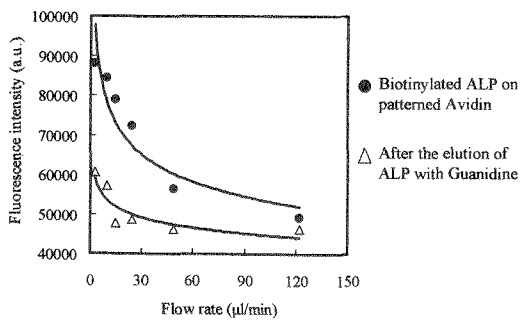

Fig. 6 Fluorescence intensity vs flow rate.

4. CONCLUSIONS

An amine reactive group could be successfully immobilized on a specific position of the micro channel inner wall using photochemical reaction, and then proteins were bound to the exact place. ATFB-SE was used as a photo-reactive crosslinker, avidin was immobilized on the surface of polystyrene microchannel, and then biotinylated enzyme was bound to the avidin. The result of the microscope fluorescence analysis supported the immobilization of proteins. It was found that cleaning the microchannel with 6 M guanidine hydrochloride salt solution suppressed non-specific binding of protein. The techniques developed in this study will be useful for immobilizing the functional proteins regioselectively on the inner surface of microchannel.

5. REFERENCES

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