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Efficient Biotinylation of Nitrocellulose Membrane for Immuno-Filtration Capture Assay

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Abstract : We investigated biotinylation of nitrocellulose membrane for immuno-filtration capture assay. In order to enhance the efficiency of biotinylation, nitrocellulose membranes were pretreated with several chemicals for the purpose of suitable protein absorption through surface modification. As a signal generating enzyme, urease was used and the concentration of avidin was optimized for the efficient binding kinetics between urease-biotin in liquid phase and biotinylated membrane in solid phase. For effective biotinylation, bovine serum albumin-biotin complexes could be immobilized at a concentration of 370 μg/stick (4.4 cm^2). Among tested chemicals, polylysine (0.25%) showed a significant effect in biotinylation. Polylysine is thought to enhance surface area by extending unbound residues into solution. Time of treatment over 30 min and higher molecular weight of polylysines (58,100 dalton) showed positive effect on the enhancement of biotinylation. The result from this study may be useful for developing a new biosensor and other biofunctional membranes for examining molecular recognition.
Key words : biotinylation, light addressable potentiometric sensor, polylysine

Bioanalytical devices, especially biosensors, have been actively studied recently due to several attractive features such as selectivity, sensitivity and small size (Byfield and Abuknesha, 1994; Rajinder, 1994). Most of the research strategies focused on the selection of the targeted biosensing materials and an efficient signal transformer. Nowadays, several type of biosensors are already available in commercial markets such as enzyme-electrode based catalytic sensor and antigen-antibody-based affinity sensor. Novel biosensors include surface plasmon resonance (SPR), resonant mirror (RM), and light addressable potentiometric sensor (LAPS) (Lukosz, 1991; Helen *et al.*, 1994; Luc and Parce, 1994).

For kinetic analyses, the properties of biochemical reactions in test tube are very important but the molecular recognition of supra-macromolecules between different phases e.g. solid-liquid and liquid-liquid phases should be studied (Michael and Ulrich, 1991).

Surface density of sensing materials could be modulated in a given surface area. If possible, maximizing the density is desirable for the enhancement of signal amplification. Also topological orientation of the active site towards the liquid phase could be controlled during the immobilization onto the solid phase (Wolfgang, 1995).

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The surface of proteins is heterogeneous, and normally exposes positive and negative charges, groups with hydrogen bonding abilities, as well as non-polar regions. Each type of protein can interact with other molecules and surfaces in a great number of ways due to the complexity of the protein surface. The most important driving forces for protein adsorption are often regarded to be hydrophobic and ionic interactions, combined with an entropy gain caused by conformational changes of the protein during adsorption (Per *et al.*, 1995).

We are much interested in the immuno-filtration capture-based immunoassay, especially in profiles of protein adsorption on nitrocellulose membrane through the modification of surfaces. We used LAPS device as a model system due to the distinct characters of the immuno-ligand assay system.

A light addressable potentiometric sensor is a silicon-based biosensor (Fig. 1.), and it was recently developed as a highly sensitive device for measuring pH, redox potentials, or transmembrane potentials resulting from biochemical reactions. This insulated semiconductor device responds to surface potentials at the eletrolyte-solid interface through the effect of such potentials on electric fields within the semiconductor. The semiconductor produces a transient photocurrent in response to transient illumination via light emitting diodes (LED). The amplitude of the photocurrent can be varied in response to

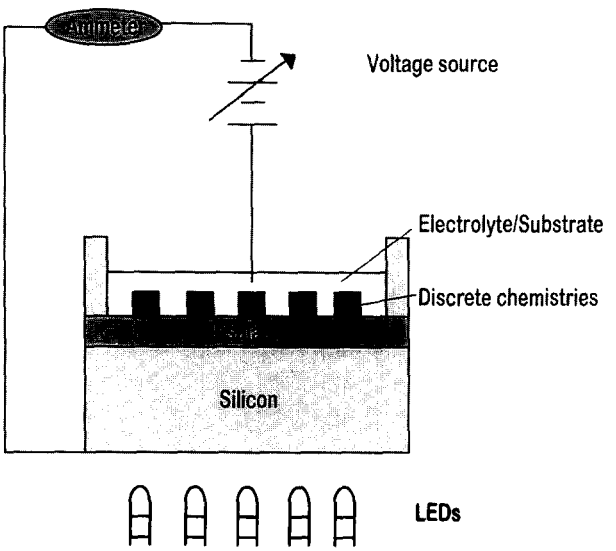

Fig. 1. Schematic representation of the structure of the LAPS.

the electric potential generated by a voltage source or the pH change of electrolyte solution.

Biomaterials of selective affinity (e.g., antibodies, receptors, or nucleic acids) can be detected if they are linked to enzymes which generate the potentiometric signal. LAPS can address different regions of the surface of semiconductor with the light of LED and multiple potentiometric measurement can be allowed simultaneously (Dean *et al.*, 1988).

Other attractive features of Threshold unit (trade name of LAPS manufactured by Molecular Device Corporation) is immuno-ligand assay system (Fig. 2.). This system consisted of two indirect capture methods i.e. fluorescein and avidin-mediated adsorption and filtration stage with the biotinylated membrane. Fluorescein-mediated capture method was designed in order to make the assay more easy and versatile in various samples with a signal-generating enzyme, urease. The avidin-mediated capture method makes the assay heterogeneous mode and reduces the non-specific binding.

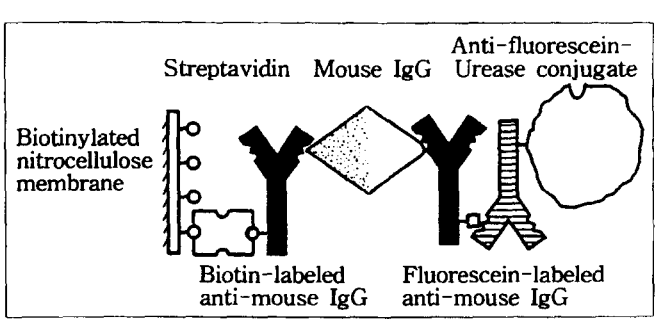

Fig. 2. Schematic representation of the reagent system of filtration capture immunoassay.

It also has the capability of concentration through the membrane. In a brief description of the assay procedure, biotin-labeled antibody, fluorescein-labeled antibody and antigen of sample are mixed and reacted for a given time at room temperature. Then streptavidin and urease-labeled anti-fluorescein antibody are added. After filtration through the biotinylated membrane which is attached in a filter holder, the membrane stick is inserted into the detection chamber which is filled with urea solution as substrate for signal generation. LAPS signal can be finally measured in units of μV/sec by contacting the membrane stick onto the sensing surface of LAPS tightly with plunger (John *et al.*, 1990).

We investigated the efficient method of biotinylation of the nitrocellulose membrane to increase the immobilized amount of bovine serum albumin-biotin complex through the modification of the membrane after preteatment with several chemicals.

Materials and Methods

Materials

Dinitrophenol-biotin-N-hyrdoxysuccinimide (Biotin-NHS) and biotinylated nitrocellulose membrane (0.45 μm pore) were obtained from the Molecular Devices (Menlo park, USA). Urease, avidin, urea and all other chemicals were purchased from the Sigma (St. Louis, USA). Nitrocellulose membranes were the Nitropure (0.45 μm pore) of MSI (Westboro, USA). As the bioanalytical device of immunoassay, LAPS was used which was manufactured by the Molecular Devices company under the Threshold trade name.

Labeling of biotin on urease and bovine serum albumin

For the labeling of biotin on urease, 1 mg of urease was dissolved in 1 ml of 10 mM PBS (pH 7.4). The molar ratio of urease to biotin-NHS was adjusted to 1:75 by addition of the biotin-NHS solution (27 μl) dissolved in anhydrous dimethylformamide (5 mg/ml) into the urease solution. The mixed solution was subsequently incubated in dark condition at room temperature. After 2 h reaction, the unbound biotin-NHS and biotin-urease complex were separated with the elution of PBS solution through the desalting column.

One milligram of bovine serum albumin (BSA) was dissolved in 1 ml of 10 mM PBS (pH 7.4) for the labeling of biotin on BSA. The molar ratio of BSA to biotin-NHS was adjusted to 1:10 by addition of the biotin-NHS solution (21 μl) dissolved in anhydrous dimethylformamide (5 mg/ml) into the BSA solution. The other procedures are same as the case of urease.

Biotinylation of nitrocellulose membrane

Nitrocellulose membranes were attached onto the plastic filter holder with thin film adhesive tape for the application to LAPS. In order to modify the surface character of the membrane, several chemical modifiers were pretreated, such as 10% methanol, 1% polyvinylpyrrolidone, 1% Triton X-100, 1% Tween 20 and 1% polylysine (MW 58100) in 10 mM PBS (pH 7.0). After washing with the distilled water and drying, 0.5 ml of biotinylated BSA solution in 10 mM PBS (pH 7.0) was impregnated and then dried. The unbound biotinylated BSA on membrane was removed by washing using LAPS filter unit.

Measurement of the amount of the immobilized biotinylated BSA

One milligram of avidin solution and 100 μl of biotinylated urease solution were mixed and then filtered at a flow rate of 100 μl/min through the biotinylated nitrocellulose membrane which was inserted in the LAPS filter block. After adhesion of the conjugate, i.e. avidin-biotinylated urease, to the biotinylated nitrocellulose membrane, the residual of the unbound conjugate was removed by washing with 2 ml of wash buffer (10 mM PBS, pH 6.5, 0.05% Tween-20) at the flow rate of 200 μl/min. The amount of the conjugate on the membrane was measured with the Threshold unit. The conjugate-loaded plastic filter holder was inserted into the detection chamber of Threshold unit which was filled with urea solution (100 mM in wash buffer) as a substrate. With the plunger, the reacted membrane can be contacted onto the sensor surface of LAPS. The relative concentration of the biotinylated BSA immobilized on membrane could be compared with the LAPS signals (μV/sec) of each test group.

Results and Discussion

Optimization of concentration of the urease-biotin conjugate

The avidin-biotin complex has been used as an extremely versatile and general mediator in a wide variety of bioanalytical applications due to the exceptional high affinity and consequent stability of this non-covalent interaction (Neir and Edward, 1988).

Filtration-capture immunoassay of LAPS is also basically used with the mediated interaction of avidin-biotin. For the purpose of having good biochemical functionality of biotinylated membrane, we studied the interaction between the solid phase (membrane)-biotin and liquid phase (buffer solution)-avidin.

Urease was biotinylated as follows. The initial molar ratio of urease to biotin was 1:75 in the reaction solution. After 2 h incubation at room temperature and elution through the desalting column, the number of biotin bound to urease was calculated.

The molecular weight of urease is 483,000 and the extinction coefficient (1 mg/ml) was measured as 0.7285. Based on the measured absorbances at 280 nm and 362 nm, the number of biotin bound to urease was calculated to be about 20 from the following formula.

$$\text{Molar Biotin/Urease} = \frac{(58 \times A_{362})(\varepsilon(\text{mg/ml}) \times \text{MW of protein}/10^6)}{A_{280}-(0.32 \times A_{362})}$$

$$\text{Urease-biotin (mg/ml)} = \frac{A_{280}-(0.32 \times A_{362})}{\varepsilon(\text{mg/ml})}$$

Prior to the manufacture of new biotinylated membrane, we have optimized the concentration of urease-biotin conjugate using the biotinylated membrane of Threshold Devices as control group. Avidin was dissolved in the assay buffer (10 mM PBS, pH 7.0, 0.05% Triton X-100) at 1 μg/ml and then 100 μl of the sequentially diluted solutions of the biotinylated urease were added. After thorough mixing, the reaction solution was filtered at the flow rate of 100 μl/min through the biotinylated membrane on plastic filter holder of Threshold unit for adsorption of the conjugate onto the membrane, and the membrane was washed with 2 ml of wash buffer (10 mM PBS, pH 6.5, 0.05% Tween-20). The results obtained from the above experiment are shown as LAPS signal versus concentration in Fig. 3.

With the increase in the concentration of urease-biotin conjugate, LAPS signal was increased linearly. But 1 μg/ml was used as the optimal concentration of the urease-biotin conjugate in order to reduce nonspecific binding and to obtain good linearity.

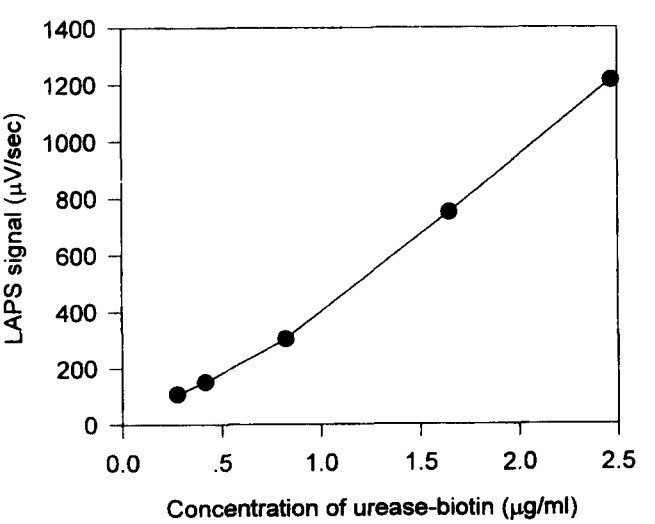

Fig. 3. Determination of optimum concentration of urease-biotin.

Determination of optimal concentration of avidin

The concentration of avidin could exert a significant effect on the binding kinetics of the filtration-capture step in a given concentration of urease-biotin conjugate at the unit surface of the uniformly biotinylated membrane. The optimal concentration of avidin was determined. Varying concentrations of avidin (1 ml) in assay buffer were mixed with 100 μl of urease-biotin solution (1 μg/ml) in assay buffer.

The mixed solutions were filtered at the flow rate of 100 μl/min through the biotinylated membrane at the LAPS filter block in order to load the conjugate on the membrane, after which the membrane was rinsed with 2 ml of the wash buffer. The LAPS signal obtained from the test is shown in Fig. 4. The LAPS signal increased with increasing concentration of avidin, but it did not show linear increment, showing the saturation above avidin concentration of 0.625 μg/ml. It is thought that too high concentration of avidin could have inhibitory effect on the mediatory binding reaction between urease-biotin conjugate and biotinylated membrane because of the excess unbounded avidin molecules. Thus the optimal concentration of avidin was determined as 0.5 μg/ml and the experiments followed thereafter were performed with this concentration.

Preparation of the biotinylated membrane

BSA was biotinylated with biotin-NHS and the molar incorporation ratio of biotin on BSA was determined as 3.8. The biotinylated membrane was prepared using the nitrocellulose membrane (MSI company) and biotinylated BSA. One half milliliter of BSA-biotin (0.47 mg/ml) dissolved in PBS (pH 7.0, 10 mM) was impregnated onto the membrane which was placed on a clean glass plate or filter holder. After drying at room temperature.

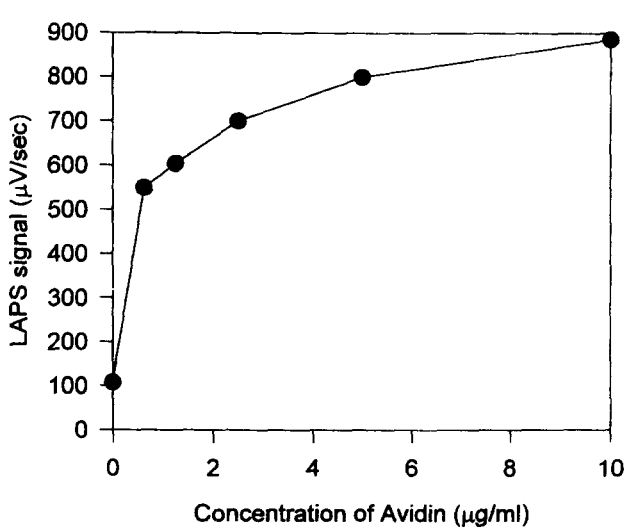

Fig. 4. Determination of optimum concentration of avidin.

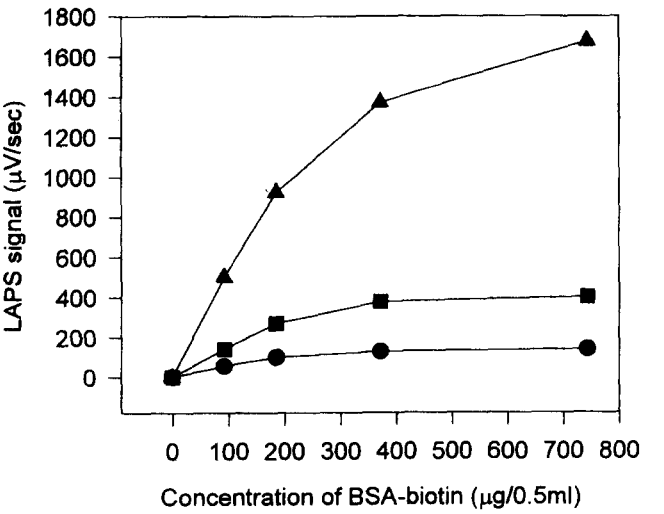

Fig. 5. Detection of optimum concentration of BSA-biotin Urease-biotin: 27.4 ng ●, 82.3 ng ■, 247 ng ▲.

the membranes were washed with buffer. The amount of immobilized biotinylated BSA was slightly higher when it was dried on the filter holder compared to that dried on the clean glass plate. It was considered that loss of biotinylated BSA occurred due to the contact of the membrane with the glass plate. Thus nitrocellulose membrane was firstly attached to filter holder with thin adhesive film tape and biotinylated on it.

We investigated the immobilized quantity of the BSA-biotin on the unit surface of nitrocellulose membrane. Varing concentrations of BSA-biotin were applied on the nitrocellulose membrane and the amount of biotin-BSA on membrane was determined using LAPS. One milliliter of avidin (0.5 μg/ml) was mixed with 100 μl of urease-biotin conjugate in the assay buffer and the mixture was filtered through biotinylated membrane, after which LAPS signal was measured. The result is shown in Fig. 5. At the three different concentrations of urease-biotin conjugate, BSA-biotin was saturated at the concentration of 400 μg/0.5 ml and the surface area of the membrane was 4.4 cm^2 (2.1×2.1 cm). The maximum amount of protein per 1 cm^2 of our experiment was 91 μg per cm^2 of membrane and this value is very close to the maximum immobilizable amount of protein (100 μg/1 cm^2) according to the data sheet of MSI company. Thus, we carried out the biotinylation experiments with 370 μg/0.5 ml/stick of BSA-biotin.

Incubation time of urease-biotin conjugate with biotin

Avidin has a high affinity (Ka=10^{15} M^{-1}) toward biotin and is a tetrameric protein of molecular weight approximately 60.000 with each subunit capable of binding one molecule of biotin. Incubation time is a very important factor in a mediatory reaction. Too long time of

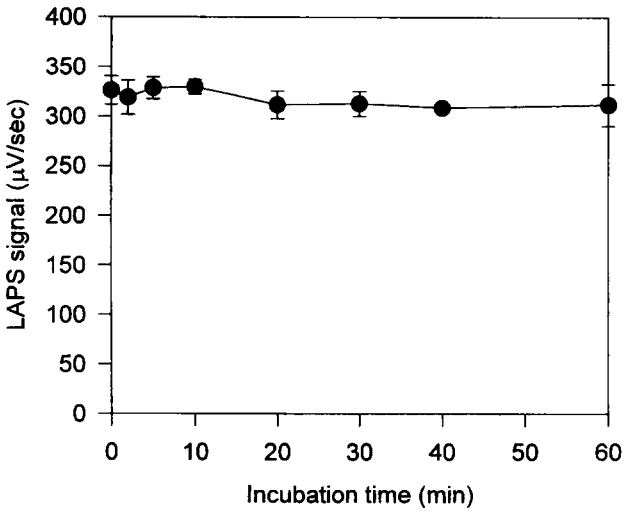

Fig. 6. Effect of incubation time of avidin and urease-biotin reaction on LAPS signal.

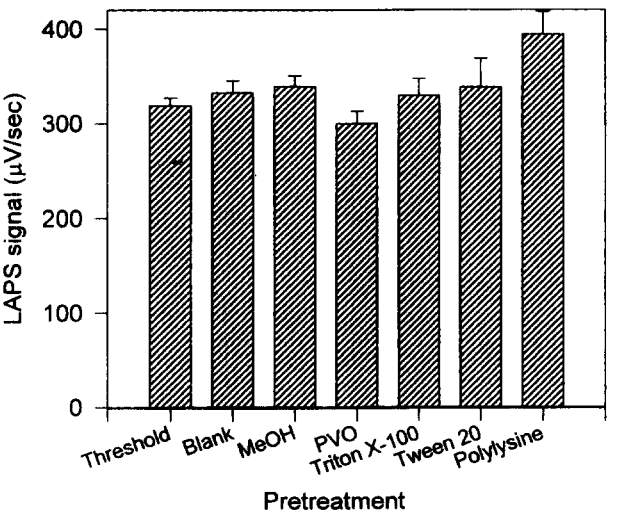

Fig. 7. Effect of pretreatment of nitrocellulose membrane on the immobilization of BSA-biotin: 10% MeOH, 1% Polyvinylpirrolidone (PVO), 1% Triton X-100, 1% Tween 20, 1% Polylysine (MW, 58100).

incubation could make the avidin bind with four molecules of urease-biotin conjugate and these complexes could not bind onto the membrane. If the incubation time is too short, avidin can not bind fully with urease-biotin and different results can be obtained. Thus we studied the effect of incubation time.

One milliliter of avidin (0.5 μg/ml in assay buffer) and 100 μl of urease-biotin (1 μg/ml in assay buffer) were mixed and reacted for various times (from 12 min to 60 min). After filtration through the biotinylated membrane, the amounts of urease-biotin were determinated as LAPS signal as shown in Fig. 6. The biotinylated membrane, used in this experiment, was prepared with the nitrocellulose membrane (MSI company) by impregnating 0.5 ml of the biotinylated BSA solution (0.74 mg/ml) in PBS (pH 7.0, 10 mM) with the subsequent drying and washing. The result shows that the effect of incubation time is almost constant through 60 minutes. It was thought that the moles of avidin was low compared to that of the biotin attached to urease.

Modification of nitrocellulose membrane

The most important driving forces for protein adsorption are often regarded to be hydrophobic and ionic interaction, combined with an entropy gain caused by conformational changes of the protein during adsorption (Ajit, 1992).

We performed the modification of the nitrocellulose membrane in order to change the surface of membrane and to enlarge the surface area by pretreatment with various chemicals, such as 10% methanol-, 1% polyvinylpyrrolidone-, 1% Triton X-100-, 1% Tween 20-, and 1% polylysine (MW 58100)-solution prepared in 10mM PBS (pH 7.0). Onto the pretreated and dried membrane, 0.5 ml of BSA-biotin (740 μg/ml) was added and dried. One ml of avidin in assay buffer (0.5 μg/ml) and 100 μl of urease-biotin solution (1 μg/ml) were mixed and filtered through the biotinylated membrane, and the amount of urease-biotin conjugate captured on membrane was measured. The resulting LAPS signal is shown in Fig. 7. The tested biotinylated membrane was more superior to the biotinylated membrane of Molecular Devices company based on the immobilized amount of BSA-biotin. The pretreatment of polylysine resulted in more BSA-biotin bound to the membrane than those of methanol, PVO, Triton X-100, and Tween-20. About 20% increase was observed in the polylysine treatment but the effect of other pretreatments was not significant.

Effect of polylysine pretreatment

The effect of polylysine was investigated at different concentrations. Each 0.5 ml of polylysine was treated onto the membrane and placed at room temperature for 1 h, after which the membrane was washed and dried. With the pretreated membrane, 0.5 ml of BSA-biotin (740 μg/ml) was immobilized, and then 1 ml of avidin (0.5 μg/ml) and 100 μl of urease-biotin (1μg/ml) in assay buffer were mixed and filtered. Fig. 8. shows the effect of polylysine concentration. The 0.25% of polylysine treatment was most effective.

The effect of pretreatment time on adsorption was also examined. The amount of adsorption increased within 30 min, but there was no further increase of adsorption up to 4 h (unpublished data).

Polylysines have a number of molecular weights and a variety of chain lengths. It was anticipated that long-

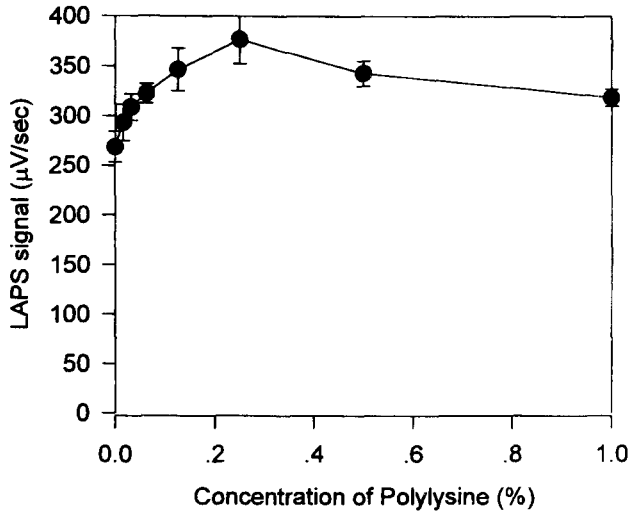

Fig. 8. Effect of polylysine (Mr=58,100) concentration on the immobilization of BSA-biotin.

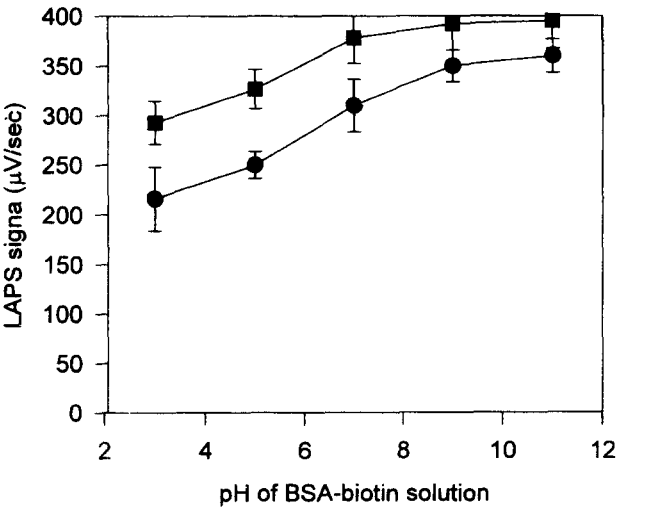

Fig. 10. Effect of pH of BSA-biotin solution on the immobilization of BSA-biotin. Polylysine-pretreated membrane ■. Blank stick ●.

er chain of polylysine would have larger surface area if the same number of molecules are attached in the same unit area. Polylysines with five different molecular weights, such as 3970, 9600, 24500, 58100, and 193700, were tested. Their concentrations were fixed as 0.25%. The result is shown in Fig. 9. The amount of immobilized BSA-biotin increased according to the increase of polylysine molecular weight, although the increment was modest.

We were also much interested in the effect of pH of BSA-biotin solution on the adsorption. It is well known that an entropy gain is caused by conformational changes of the protein during adsorption. In a harsh pH environment, protein is denatured and the interior hydrophobic region of the protein is exposed to the solution.

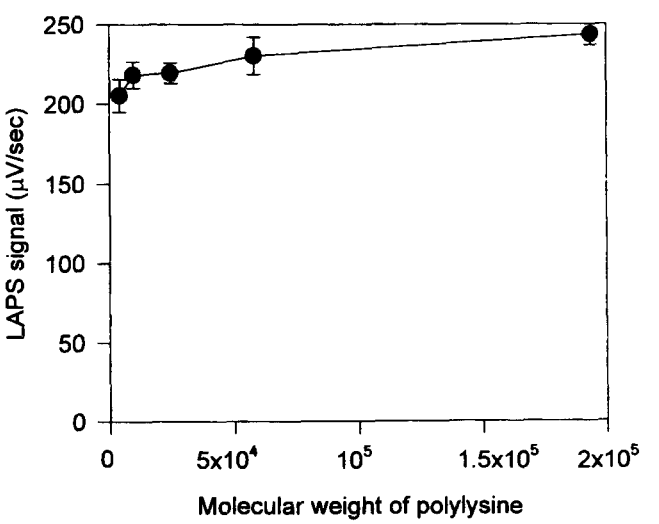

Fig. 9. Effect of molecular weight of polylysine on the immobilization of BSA-biotin.

Thus hydrophobic interaction would prevail during adsorption. In addition, the sequential change of pH can make the protein renature. With the pretreated membrane with 0.25% polylysine, 0.5 ml of BSA-biotin (0.45 μg/ml) was prepared in the buffers of pH 3.0, 5.0, 7.0, 9.0, and 11.0 respectively, and tested. The results are plotted in Fig. 10. The amount of BSA-biotin was increased in response to the increase of pH, but the increments of the immobilized amounts above pH 7 were diminished gradually. A similar result was obtained with the polylysine pretreated membrane.

We investigated the efficient method of biotinylation of the nitrocellulose membrane to increase biotinylation efficiency through the modification of the membrane with preteatment with several chemicals. Among the tested chemicals, polylysine showed a significant effect in biotinylation.

Immuno-filtration capture assay has more sensitivity than immunoassay on solid surface because membranes provide a large surface area. Hydrophilic polymer is proved to be most suitable in shielding the surface against non-specific binding and generating active sites for the attachment of ligands (Löfas *et al.*). Polylysine is expected to provide a strong electrostatic adsorption due to the multiple charge of its structure (Claire, *et al.*, 1994) and to enhance surface area by extending unbound residues into solution. Furthermore polylysine has many active sites for conjugation with protein. Thus result of the experiment with polylysine suggests potentiality of polylysine as a good treatment agent to increase protein binding on surface for immunoassay. Systematic integration of pretreatment for surface activation and attachment of functionalized biomaterials should be studied in future research.

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