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Fiber-Optic Ethanol Sensor Using Alcohol Dehydrogenase-Immobilized Langmuir-Blodgett Film

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A fiber-optic biosensor using alcohol dehydrogenase (ADH)-immobilized Langmuir-Blodgett (LB) films is developed for the measurement of ethanol concentration. The enzyme was immobilized on the lipid monolayers by adsorption from the aqueous subphase solution. A decrease of affinities to ethanol and NAD in ADH-immobilized LB films is observed compared with those of free enzyme based on ordered multisubstrate kinetics. From the measurement of the surface pressure-area isotherm and retained enzyme activity for the four types of lipids, we determined that negatively charged arachidic acid is the most suitable for adsorption of ADH, based on its electrostatic force as well as hydrophobicity, compared to other lipids. The adsorption isotherm of ADH on the lipid monolayer is a Langmuir isotherm, which indicates that the extent of ADH coverage on the lipid layer is less than one complete monolayer. The sensor output shows a linear relationship with ethanol concentration up to 40 mM when the ADH-immobilized LB film has 20 layers. As the number of ADH-immobilized LB filmlayers is increased to 20, the linear response range increases. However, the increase in output signal intensity is not proportional to the increase in the number of ADH-immobilized LB films and the increase in the detection range is limited when the ADH-immobilized LB film has 20 layers.

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1. Introduction

Ethanol sensors may be useful for application in various industrial fields, such as the fermentation industry, the pharmaceutical industry, and the beverage industry, for the diagnosis and control of processes.[1] Since an enzyme shows specific catalytic activity towards a substrate, the enzyme recognizes the molecules and reacts with them. Biosensors utilizing enzyme reaction are simple, require no expensive equipment, and can simultaneously measure numerous samples. Therefore, many biosensors have been developed for transducer, pH electrode, immunoassay, carbon paste electrode (CPE), optics and ion-selective field-effect transistor (ISFET) applications.[1,2] Biosensors for the detection of ethanol have been extensively researched.[1,3] The detection principle of these sensors is based on the reactions of alcohol dehydrogenase, alcohol oxidase or catalase with ethanol.

Fiber-optic sensors using optics as the transducer and optical fiber as the light guide have many advantages compared to other sensor types due to their remote sensing capability and the possibility of multiplexing many sensors to a central instrument.[1-3] Fiber-optic biosensors are an emerging technology that incorporate sophisticated biological and optical components for analysis of specific components with high sensitivity and selectivity.

The Langmuir-Blodgett (LB) technique has been used to fabricate an organized molecular film for use in the sensor device. The immobilization of enzyme molecules on the lipid monolayers has been attempted for chemical sensors.[4-9] The advantages of this technique are ultrathin film deposition, highly ordered molecular array, ease of packing and stacking molecules, low temperature and biomimetic membrane fabrication. Due to the formation of a highly ordered enzyme layer on the molecular level, one-step fabrication of the active layer and an ultrathin layer, which leads to short response times compared to other enzyme immobilization methods, the LB technique has been used to immobilize the enzymes in the biosensor.[4,10] Several studies have been performed to demonstrate the feasibility of biosensors using the LB technique.[4-11] However fiber-optic biosensors using ADH-immobilized LB films for the detection of ethanol have not yet been developed.

In this study, a fiber-optic biosensor using ADH-immobilized LB films is developed to measure ethanol concentration. Ethanol is reacted with NAD using ADH-immobilized LB film. During the reaction, NADH, the fluorescence material, is produced and emits fluorescence at 455 nm in response to excitation light of 340 nm. To detect the fluorescence, NADH is excited by light from a nitrogen laser and the emitted light is delivered to the photodiode array through the fiber-optic bundle. Ethanol concentration can be determined from the intensity of NADH-dependent fluorescence.

To evaluate the extent of reaction of ADH-immobilized LB film quantitatively, the kinetic parameters in the ADH catalytic reaction are obtained based on ordered multisubstrate kinetics. To understand the mechanism of enzyme adsorption, the effects of surface charge and hydrophobicity of the lipid layer on the adsorption of ADH are investigated by measuring the surface pressure-area isotherm and the retained enzyme activity of ADH-immobilized LB films. The adsorption kinetics of enzyme on the lipid monolayer is measured to evaluate the monolayer coverage of enzyme on the lipid layer and to determine the optimum amount of enzyme to be supplied during the formation of the LB film.

2. Materials and Methods

2.1 *Materials*

Alcohol dehydrogenase (ADH) (370 units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO.). Stearyltrimethylammonium chloride ($C_{18}N$) was purchased from Tokyo Kasei Chemical (Tokyo, Japan). All other chemicals were of reagent grade and were purchased from Sigma Chemical Co.

2.2 *Enzyme immobilization*

HEPES buffer of 1 mM is used as the subphase at pH 7.0, which is adjusted with 1 M NaOH solution. Four lipids (C_{20}, $C_{20}Me$, $C_{18}N$ and C_{18}) are dissolved in chloroform to make 1 mM, which are used to form the monolayers. $C_{18}N$ is mixed with $C_{20}Me$ (molar ratio of 1:4) to form a monolayer of $C_{18}N_{0.2}$.[3] The surfaces of solid substrate, quartz and slide glass are made hydrophobic by dipping in 0.2% n-octadecyltrichlorosilane toluene solution for 30 min and then thoroughly rinsing with fresh toluene.

The ADH-immobilized lipid monolayer is prepared according to Formherz's method.[4,12] The multicompartment trough made by Nima Tech. (Coventry, England) is used. The prepared lipid solution is spread and left for 20 min in one compartment. After evaporation of chloroform, the lipid monolayer is formed and is then compressed to a surface pressure of 20 mN/m. The enzyme solution, prepared by dissolving the solid enzyme in the same buffer, is injected into another compartment. The lipid monolayer, keeping a constant area, is moved to the enzyme-containing compartment. The lipid monolayer is left for 60 min for the adsorption of enzyme molecules onto the lipid monolayer. After that, it is expanded to a pressure of 10 mN/m and then left for 5 min. The monolayer is recompressed to 20 mN/m. After forming the ADH-immobilized lipid monolayer, it is transferred from the enzyme-containing compartment to the dipping compartment and dipping at a speed of 5 mm/min is performed.

2.3 *Enzyme kinetics*

Ethanol is reacted with NAD in the presence of ADH, as shown in eq. (1).[2,13] To investigate enzyme kinetics, ethanol is dissolved in phosphate buffer at pH 8.8 with NAD. Free ADH and 20-layer ADH-immobilized LB film are used. NADH produced via the following equation is measured by fluorescence.

$$\mathrm{NAD} + \mathrm{C_2H_5OH} \xrightarrow{\mathrm{ADH}} \mathrm{NADH} + \mathrm{CH_3CHO} \qquad (1)$$

2.4 *Construction of a fiber-optic biosensor*

The schematic diagram for the experimental system is shown in Fig. 1. When light from a nitrogen dye laser (Laser Science Inc., Newton, MA) excites the NADH produced in the reaction, NADH-dependent fluorescence is transmitted to the photodiode array detector (Oriel Co., Stanford, CT) through the fiber-optic bundle (Oriel Co., Stanford, CT). The detector converts the optical output signal to an electrical output signal which is delivered to a computer for analysis.

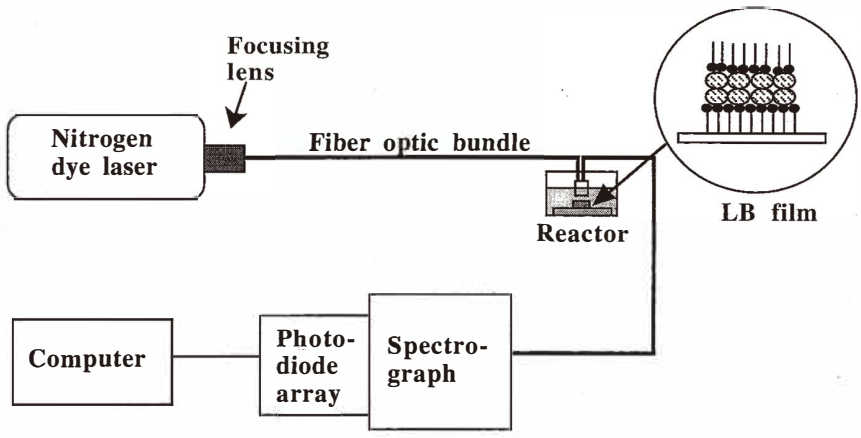

Fig. 1. The setup of a fiber-optic biosensor.

2.5 *Activity measurement*

The substrate with the ADH-immobilized LB film is immersed in a phosphate buffer solution with 7.5 mM NAD and 15 mM ethanol. The activity of the ADH-immobilized LB films is estimated by measuring NADH-dependent fluorescence.

2.6 *Adsorption isotherm*

Various amounts of ADH solution are injected to the subphase, 1 mM HEPES buffer solution, in the compartment of the LB trough. ADH-immobilized LB films are deposited on a quartz substrate. To evaluate the amount of enzyme in the ADH-immobilized LB film, o-phthalaldehyde and 2-mercaptoethanol are reacted with ADH. A complex of o-phthalaldehyde and the amino group of ADH, the fluorescence material, is formed.[4] Since the complex fluoresces at 455 nm in response to excitation light of 340 nm, the fluorescence intensity at 455 nm is measured to estimate the amount of adsorbed ADH.

3. Results and Discussion

3.1 *Enzyme kinetics*

The ordered multisubstrate mechanism is applied to the ADH catalytic reaction with two substrates (NAD and ethanol) since binding of NAD with ADH induces a conformational change which exposes a site for subsequent ethanol binding.[7] The ternary complex does not accumulate in the steady state but is rapidly converted to the ternary complex product and then to the ADH-NADH binary complex.[13] Based on the above observation, the reaction scheme of ADH can be written as eq. (2).

$$\begin{array}{ccc} E + A \leftrightarrow EA & & EC \leftrightarrow E + C \\ + & & + \\ B & & P \\ \updownarrow & & \updownarrow \\ EAB & \leftrightarrow & EPC, \end{array} \tag{2}$$

where E is ADH, A is NAD, B is ethanol, P is acetaldehyde and C is NADH. The above type of kinetic mechanism is known as the ordered Bi-Bi system in multisubstrate kinetics.[14]

A steady-state derivation yields

$$\frac{v}{V_{max}} = \frac{C_A C_A}{K_{ia} K_{mB} + K_{mB} C_A + K_{mA} C_B + C_A C_B}, \tag{3}$$

where v is reaction velocity, V_{max} is maximum reaction velocity, K_{ia} is dissociation constant of A for ADH, K_{mA} is concentration of A which yields half-maximum velocity upon saturation of B, K_{mB} is concentration of B that yields half-maximum velocity upon saturation of A, C_A is concentration of NAD and C_B is concentration of ethanol.

If the film is very thin and the reaction rate is very fast in the immobilized enzyme layer, the effect of the internal diffusion of substrates and the reaction product in the immobilized enzyme layer can be neglected.[15] Since the thickness of 20-layer enzyme-immobilized LB film is assumed to be less than 0.2 mm,[10,16] the internal diffusion effect can be neglected. Thus the derived free enzyme kinetics could be used to analyze the reaction of immobilized enzyme in a completely mixed reactor system. In Fig. 2(a), experimental data are shown in a $1/v$ versus 1/B plot for 20-layer ADH-immobilized LB film with 25 mM or 7.5 mM NAD. Ordered Bi-Bi multisubstrate kinetics describes the experimental result. The values of V_{max} and K_{mA} are obtained using the value of 1/B at the intercept with the $1/v$ axis. The calculated V_{max}, K_{mA} and K_{mB} are 0.011 mM/s, 9 mM and 200 mM, respectively, for immobilized enzyme.

For the free enzyme at 25 mM and 7.5 mM NAD, the calculated V_{max}, K_{mA} and K_{mB} are 0.023 mM/s, 1 mM and 13 mM, respectively, using data taken from Fig.2(b). In immobilized ADH, V_{max}, is lower and K_{mA} and K_{mB} are higher compared with those of the free enzyme. The above results suggest that affinities of ADH in the LB film to NAD and ethanol are reduced to 11.1% and 6.5% of those of the free enzyme, respectively. This result is likely to be due to the orientation and structural change of the enzyme in the LB film, which reduces affinities of enzyme.

3.2 *Surface-area pressure isotherm*

To investigate the surface charge and hydrophobicity effect on enzyme adsorption, π-A isotherms are obtained for the pure lipid monolayers and the ADH-immobilized lipid monolayer. π-A isotherms of four kinds of lipid monolayers and ADH-immobilized lipid monolayers on the subphase in the LB trough are shown in Figs. 3(a) to 3(d). In Fig. 3(a),

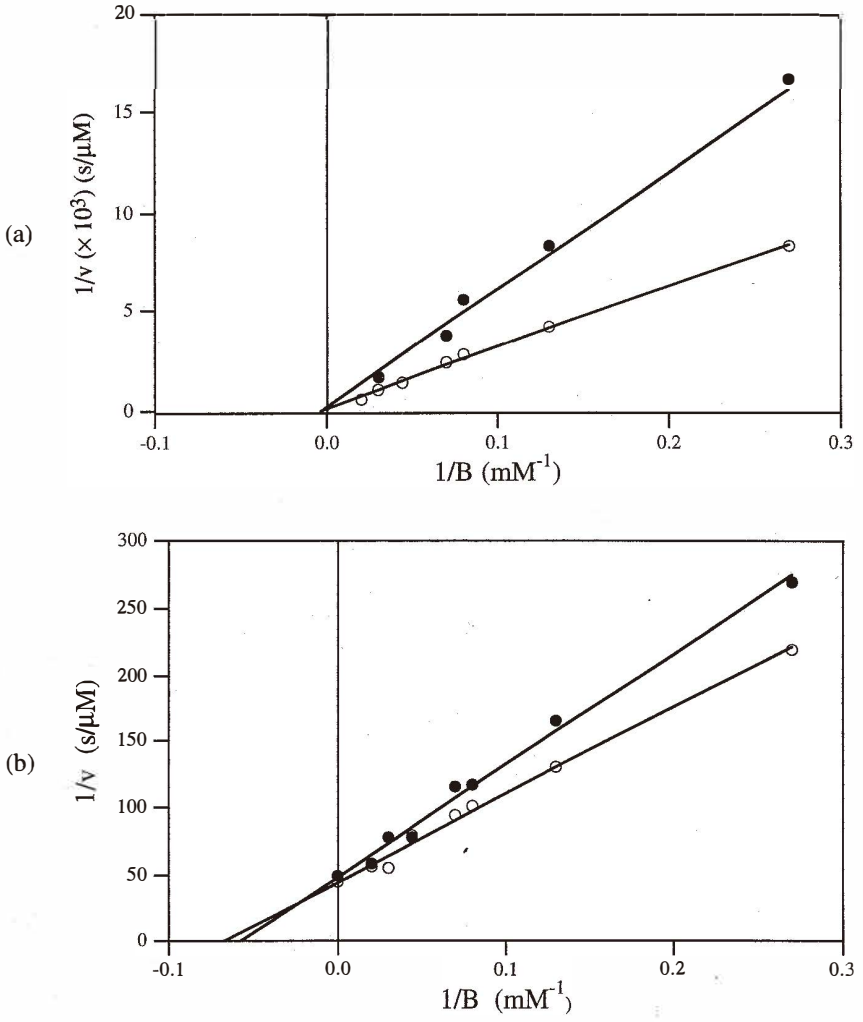

Fig. 2. (a) Double reciprocal of $1/v$ plotted against 1/B for 20-layer ADH-immobilized LB film. (b) Double reciprocal of $1/v$ plotted against 1/B of experimental data of free ADH. (○: 25 mM NAD and ●: 7.5 mM NAD)

the areas per molecule of C_{20} and ADH-C_{20} film are 25 Å^2 and 52 Å^2, respectively. In Fig. 3(b), the areas per molecule of C_{20}Me and ADH-C_{20}Me film are 24 Å^2 and 45 Å^2, respectively. In Fig. 3(c), the areas per molecule of $C_{18}N_{0.2}$ and ADH-$C_{18}N_{0.2}$ film are 23 Å^2 and 42 Å^2, respectively. In Fig. 3(d), the areas per molecule of C_{18} and ADH-C_{18} film are 20.5 Å^2 and 30 Å^2, respectively. For the ADH-immobilized lipid monolayer, the distinc-

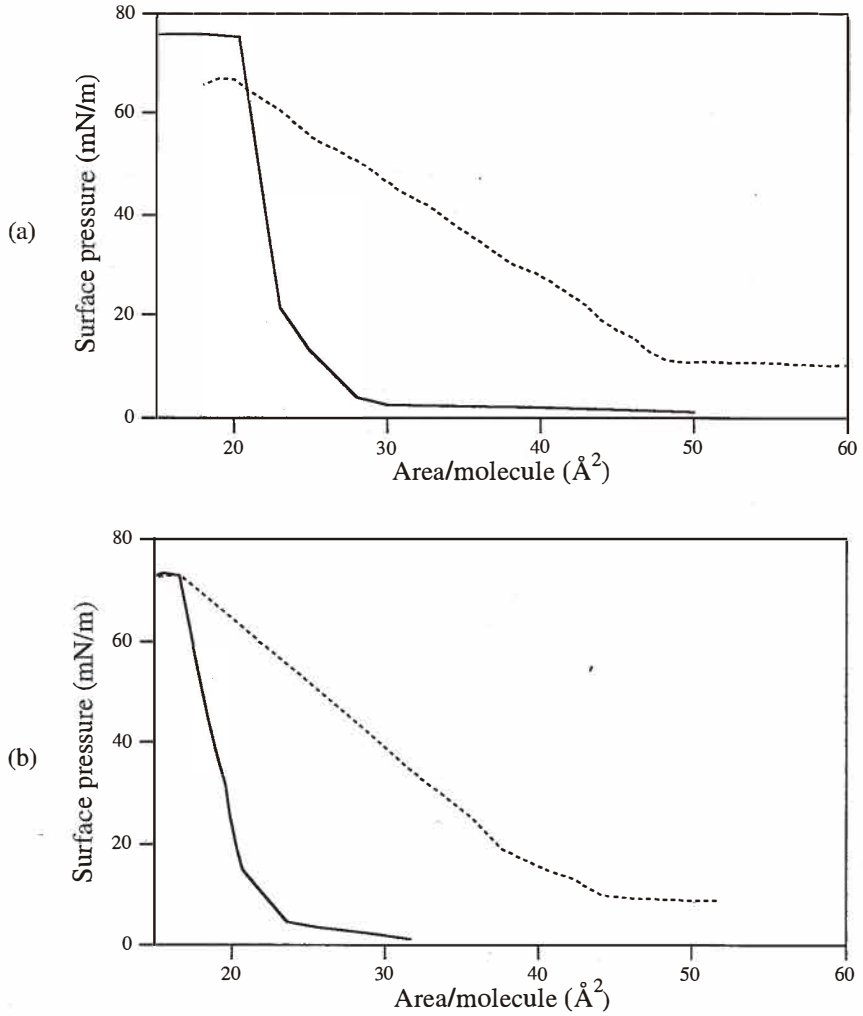

Fig. 3. Pressure-area isotherms of lipid monolayers before and after ADH adsorption. (a) arachidic acid, (b) arachidic acid methylester.

tion between the phase regions is unclear and the area per molecule is larger than that of the pure lipid monolayer. The area per molecule of the pure lipid monolayer is less than that of the ADH-immobilized lipid monolayer. These data indicate that adsorption of ADH on the lipid molecules induces an increase in area per molecule. This result may be due to the adsorption of enzyme molecules on and their permeation into the lipid monolayer. A similar result was observed for the adsorption of glucose oxidase on a lipid monolayer.[4,5]

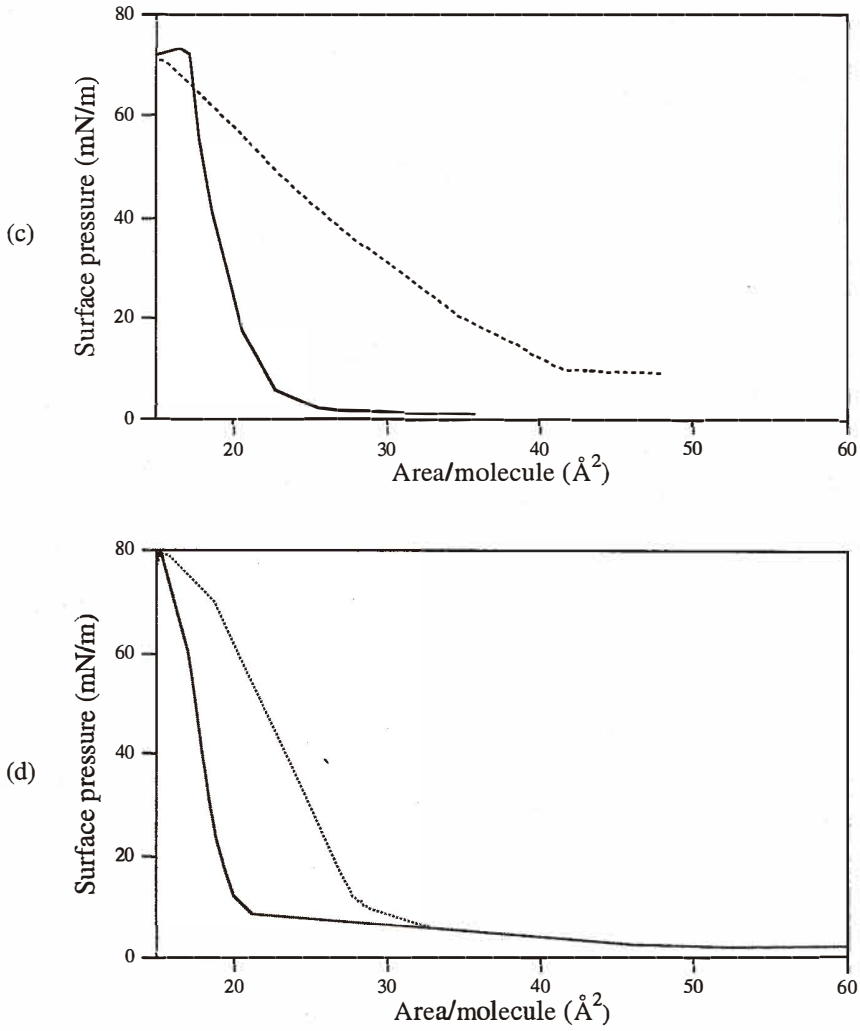

Fig. 3. Pressure-area isotherms of lipid monolayers before and after ADH adsorption. (c) stearytrimethylammonium chloride, (d) stearic acid (line: lipid before ADH adsorption, broken line: lipid after ADH adsorption).

The area per molecule of ADH immobilized on arachidic acid is the largest compared with those on the other lipids. This is likely to be due to the electrostatic force acting between the ADH molecule and the arachidic acid molecule. Since the isoelectric point of ADH is about 8.08,[17] ADH has a positive charge at pH 7.0. At pH 7.0, the surface charges of C_{20}, $C_{20}Me$ and $C_{18}N_{0.2}$ are negative, neutral and positive, respectively. When ADH is

adsorbed onto the lipid layer, the enzyme with positive charge is more strongly attracted by arachidic acid with negative charge than by lipids with positive or neutral charge. This result is consistent with the adsorption of negatively charged glucose oxidase onto positively charged lipid reported in ref. 4.

When comparing Fig. 3(a) with 3(d), the area per molecule of immobilized ADH on stearic acid is smaller than that of arachidic acid, which indicates that a smaller amount of enzyme permeates into the lipid layers. Alhough the electrostatic charge is identical for both arachidic acid and stearic acid, the arachidic acid is more hydrophobic due to the presence of a longer carbon chain than that present in stearic acid. Since ADH has a hydrophobic property, it is likely to be more attracted to arachidic acid which is more strongly hydrophobic than stearic acid. This result suggests that the adsorption of ADH is affected by the hydrophobicity of lipids. From the measurement of the π-A isotherm, we determined that the arachidic acid is the most suitable lipid for adsorption of ADH, based on the electrostatic force as well as hydrophobicity of the four lipids.

3.3 *Effect of surface charge on activity of ADH-immobilized LB films*

Table 1 shows a comparison of the activities of the three lipids in 10 layers of ADH-immobilized LB film. It is found that the C_{20} monolayer has the highest activity compared with $C_{20}Me$ and $C_{18}N_{0.2}$.

It is therefore confirmed that arachidic acid is the most suitable lipid for the adsorption of ADH onto the lipid layer, based on the electrostatic force. Since the charged state of the lipid monolayer and total charge of the enzyme affect the adsorption of ADH, the electrostatic force is a key factor in the adsorption of enzyme.

3.4 *Adsorption isotherm of ADH on lipid monolayer*

The adsorption isotherm is obtained in order to investigate the adsorption behavior of ADH from the aqueous subphase to the arachidic acid monolayer. The adsorption isotherm of ADH is shown in Fig. 4. The amount of enzyme adsorbed increases until the amount of injected enzyme reaches less than about 180 units. Above about 180 units, the amount of enzyme adsorbed becomes saturated. The adsorption isotherm of ADH is a Langmuir isotherm. This result indicates that the extent of ADH coverage is less than one complete monolayer on the lipid layer. It has also been found that the optimum amount of injected ADH is about 180 units under the present experimental conditions.

Table 1
Comparison of the activities of ADH-immobilized LB films.

	C_{20}	$C_{20}Me$	$C_{18}N_{0.2}$
Activity (arb. units / cm^3)	0.99	0.65	0.44

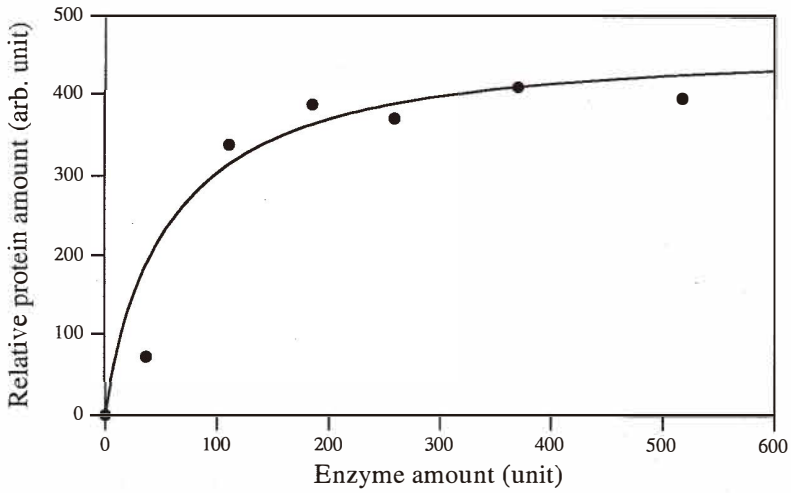

Fig. 4. Adsorption isotherm of ADH on arachidic acid.

3.5 *Sensor output analysis*

The ethanol sensor output at 7.5 mM NAD for the ADH-C_{20} LB film is shown in Fig. 5. The sensor output is proportional to ethanol concentration of up to 40 mM for 20 LB layers. This sensor output is correlated to the number of LB layers. At ethanol concentrations below 25 mM, the sensor output is saturated for 2 LB layers. However at ethanol concentrations from 25 mM to 40 mM, the sensor output is not saturated for 20 LB layers. As the number of LB layers increases, the linear response range of the sensor increases. The reason for the change in linear response range is that the amount of immobilized enzyme is increased as the number of LB layers is increased. The degree of enzymatic action of ADH at a given reaction time and a given ethanol concentration is directly related to the amount of immobilized ADH. However, the increase in output signal intensity is not proportional to the increase in the number of ADH-immobilized LB layers, as shown in Fig. 6. The output signal intensity was not significantly increased for more than 20 layers of ADH-immobilized LB film. This result might be due to a decrease in the amount of enzyme loaded, to the fact that enzyme activity is induced by the accumulation of imperfections in LB films, which are generally observed during LB film formation using organic molecules,[18] or to the orientation change of enzyme molecules, observed in double enzyme-LB layers,[10] as the number of LB film layers increases. No investigation of imperfections and orientation changes of enzyme molecules in multilayers of enzyme-immobilized LB films has yet been reported and thus should be conducted using atomic force microscopy and X-ray diffraction analysis in order to evaluate the enzyme reaction quantitatively.

From the above results, it can be assumed that the detection range of ethanol concentration could be increased by increasing the number of ADH-immobilized LB films to 20.

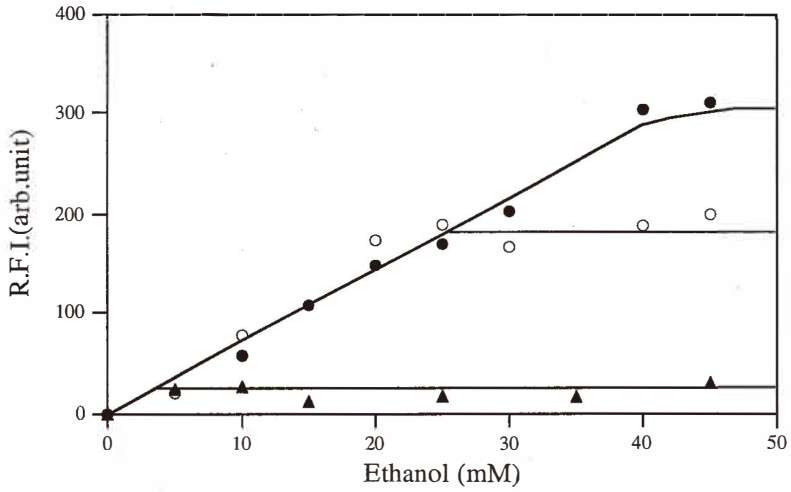

Fig. 5. Output of a fiber-optic ethanol sensor. (▲: 2 layers, ○: 10 layers, and ●: 20 layers)

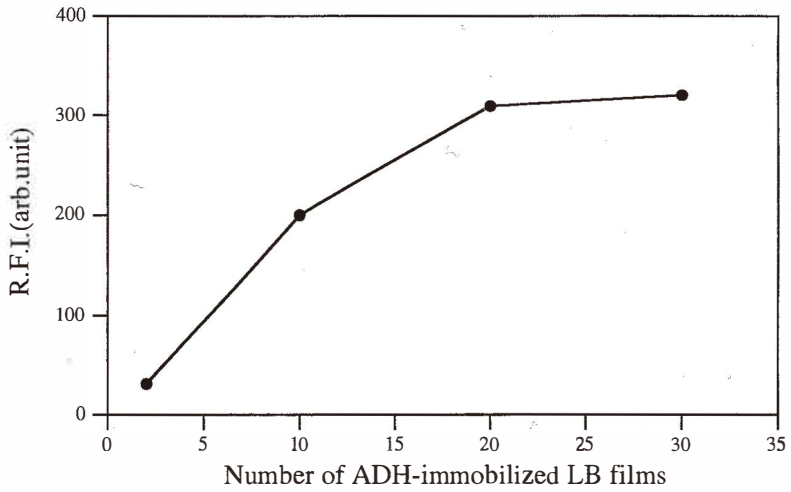

Fig. 6. The relationship between the sensor output and the number of ADH-immobilized LB film layers at ethanol concentration of 45 mM.

However, the increase in output signal intensity is not proportional to the increase in the number of ADH-immobilized LB films, and the increase in the detection range is limited when the ADH-immobilized LB film has 20 layers.

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