Enzyme Sensors Based on an Ion-Sensitive Field Effect Transistor Coated with Langmuir-Blodgett Membranes. Use of Polyethyleneimine as a Spacer for Immobilizing α-Chymotrypsin

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A highly branched polyethyleneimine (PEI) was used as a spacer for immobilizing α-chymotrypsin on the surface of Langmuir-Blodgett (LB) membranes which were deposited on the gate of an ion-sensitive field effect transistor (ISFET). α-Chymotrypsin could be covalently immobilized through the glutaraldehyde-activated PEI on the LB membrane-coated ISFET. The α-chymotrypsin-modified ISFET showed a potentiometric response to the substrate at concentrations of more than 0.1 mM. Some performance characteristics of the sensor, such as pH response, response time, and long-term stability were examined.

An ion-sensitive field effect transistor (ISFET) is widely employed for the fabrication of miniature sensors sensitive to ions and molecules.[1−10] We have already reported that a Langmuir-Blodgett (LB) membrane can be used as a support for immobilizing ionophores and enzymes on the ISFET gate.[11−13] By this technique, K^+-ion and penicillin sensors could be constructed. In the case of the penicillin sensor, penicillinase was strongly adsorbed, through electrostatic or hydrophobic force, to the surface of LB membrane. However, it is not always possible for enzymes to be adsorbed strongly on the LB membrane surface without loss of the catalytic activity. In this connection, Watanabe *et al.* have used a reactive LB membrane which possesses amino groups on the surface, for covalently immobilizing glucose oxidase on an SnO_2 electrode.[14] We have reported preliminary results on an alternative method to immobilize enzymes tightly on the LB membrane surface, in which a highly branched polyethyleneimine (PEI) is used as a reactive spacer between the enzyme and LB membrane.[15] The present paper describes in detail the performance characteristics of ISFET enzyme sensors prepared based on the PEI-activated LB membrane.

Experimental

The ISFET was fabricated on a p-silicon wafer, which was 0.5 mm wide, 6.5 mm long and 0.2 mm thick. A silicon nitride layer (1000 Å) was grown on a silicon oxide gate by the chemical vapor deposition method. The ISFET thus prepared showed a near-Nernstian response over the pH range of 1—13. The procedure for making the ISFET and the properties were reported elsewhere.[16]

Stearic acid and stearyl alcohol were used after recrystallization. Succinimidyl behenoate was synthesized from behenic acid and *N*-hydroxysuccinimide as reported.[17] PEI (MW = 40000—50000) was purchased from Tokyo Kasei Co. and used without further purification. α-Chymotrypsin was obtained from Sigma Co. L-Acetyltyrosine ethyl ester (ATEE) was purchased from Research Organics Inc.

The immobilization of α-chymotrypsin on the surface of LB membranes deposited on the ISFET gate was carried out in the following manner. We used two kinds of LB membranes to which the enzyme was immobilized *via* PEI. First, stearic acid LB membrane was deposited (20 layers) on the ISFET, from a monolayer spread on a water surface, as a Y-type multilayer.[12,13] By this treatment, the uppermost surface of the LB layer should exhibit hydrophilicity due to the carboxylic residues of stearic acid. The ISFET probe coated with stearic acid LB membrane was immersed in 0.3% PEI solution (pH 7.5) for 2 h, during which time the polymer was adsorbed to the LB membrane surface through electrostatic interaction between carboxylic groups at the surface and amino residues in PEI. The PEI-adsorbed probe was treated with 2.5% glutaraldehyde solution for 2 h to introduce aldehyde groups in the PEI. Finally, the ISFET was dipped in
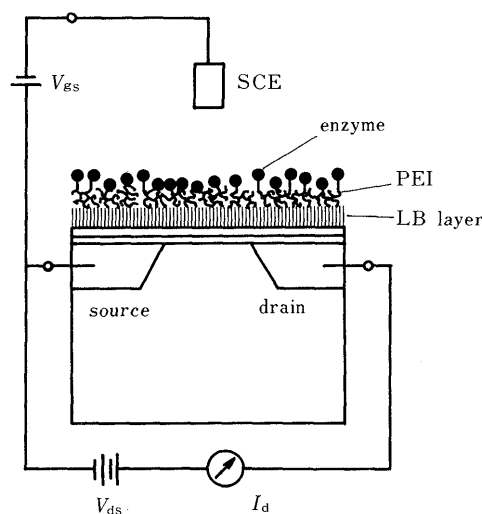

Fig. 1. A Schematic Illustration of an ISFET Modified with an Enzyme-Binding LB Membrane

0.5% α-chymotrypsin solution (2 mM phosphate buffer, pH 7.5) for 2 h, and rinsed thoroughly with the working buffer before use. The α-chymotrypsin-immobilized ISFET thus prepared is schematically depicted in Fig. 1. Also used was succinimidyl behenoate LB membrane. A mixed monolayer of succinimidyl behenoate and stearyl alcohol, which was prepared on pH 7 water at 17 °C, was deposited on an ISFET which had previously been coated with an 11-layer LB membrane composed of stearic acid. The immobilization of enzyme on the succinimidyl behenoate LB membrane was carried out using PEI similarly to the case of stearic acid LB membrane.

All measurements were conducted at 23 °C. For the estimation of long-term stability of the sensors, the potentiometric measurement was performed once a day and the probe was stored in the working buffer at 4 °C, when not in use.

Results and Discussion

Figure 2 shows the pH response of the ISFET modified with α-chymotrypsin-linked stearic acid LB membrane. The gate potential, E_{gs}, changed rapidly in the negative direction with the shift in pH of the sample solution from pH 6.0 to pH 7.0 and 7.9. The response time was within 15 s. On the other hand, positive shifts were observed when the pH value of the sample solution was changed from pH 7.9 to pH 7.0 and 6.0. The sensitivity was *ca.* 50 mV/pH, which was somewhat lower than that of the ISFET without LB membrane. These results show that, though the pH response of the ISFET is reduced to a small extent by the LB
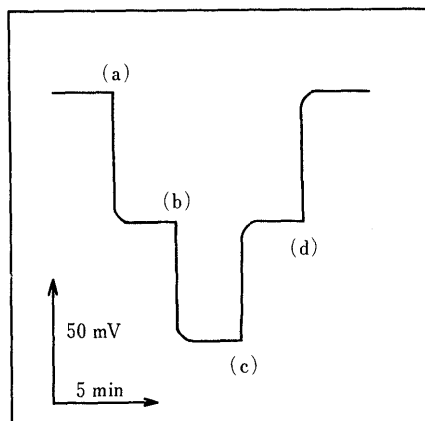
Fig. 2.   pH Response of the α-Chymotrypsin-Immobilized ISFET

The pH value of the sample solutions was changed from pH 6.0 to pH 7.0 (a), from pH 7.0 to pH 7.9 (b), from pH 7.9 to pH 7.0 (c), and from pH 7.0 to pH 6.0 (d).
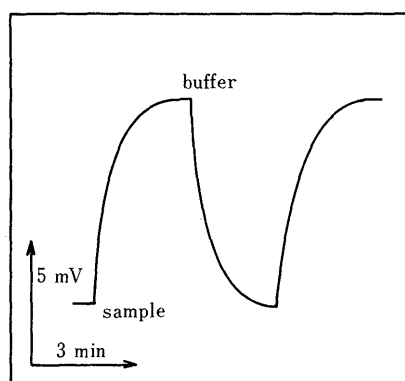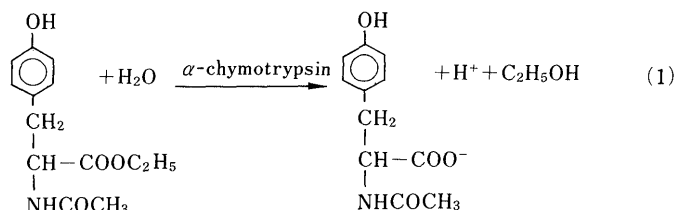

Fig. 3.   Typical Response of the α-Chymotrypsin-Immobilized ISFET to 2 mM ATEE

The 2 mM phosphate buffer (pH 8.0) was used.
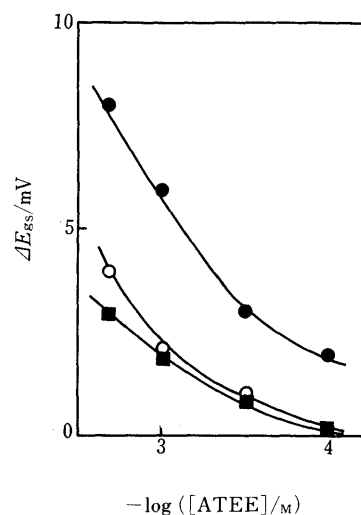

$-\log([\text{ATEE}]/\text{M})$

Fig. 4.   The Calibration Graphs of the Sensor for ATEE

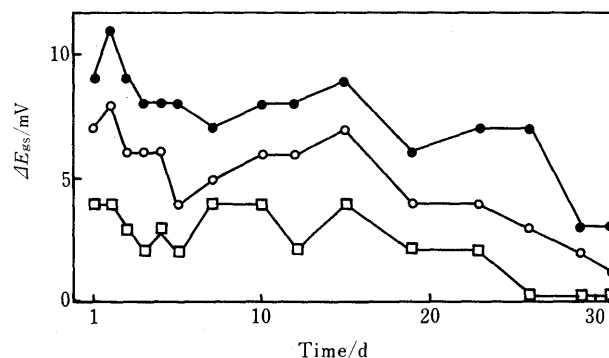The 2 mM phosphate buffer was used at pH 6.0 (—●—), 7.0 (—○—), and 8.0 (—■—).

Fig. 5.   The Long-Term Stability of the Sensor

The ΔE_{gs} values were measured for 2 (—●—), 1 (—○—), and 0.3 mM (—□—) ATEE in 2 mM phosphate buffer at pH 8.0.

membrane coating, the probe can be used as the pH-sensitive underlying device of enzyme sensors.

Figure 3 shows a typical response of the α-chymotrypsin-immobilized ISFET sensor to 2 mM ATEE at pH 8. The enzyme was immobilized on the ISFET coated with PEI-adsorbed stearic acid LB membrane. The E_{gs} value of the device was shifted positively on immersing it in ATEE solution and, by washing with the working buffer, the original value of E_{gs} was recovered. The potentiometic response (ca. 10 mV) should arise from the local pH change around the gate surface of the ISFET, which in turn originates from the hydrolysis reaction of ATEE catalyzed by α-chymotrypsin immobilized on the LB membrane (Eq. 1).

The potentiometric response of the sensor to ATEE was fairly reproducible during repeated measurements. These results clearly show that α-chymotrypsin can be immobilized tightly via glutaraldehyde-activated PEI to the LB

membrane without deactivation.

Typical calibration graphs of the sensor at pH 6, 7, and 8 are depicted in Fig. 4. The sensor showed rather low response in pH 6 and 7 media, as compared with that at pH 8. This is presumably due to the fact that the optimum pH of α-chymotrypsin is around pH 8.[18]

Figure 5 shows the reusability of the sensor. The E_{gs} values of the sensor for 2, 1, and 0.3 mM ATEE were measured once a day for 30 d. The V_{gs} value decreased gradually with time, with some day-to-day fluctuation. The fluctuation may be mainly a consequence of the small response value.

We have reported that α-chymotrypsin can be immobilized covalently through an amide linkage to the surface of LB membrane containing active ester of fatty acid.[17] However, the catalytic activity of the enzyme immobilized by this method was lost within a few days, presumably due to denaturing of the enzyme at the membrane surface. To overcome this problem, PEI has been covalently inserted as spacer between the active ester LB membrane and the enzyme. The primary amino groups in PEI can attack the active ester to form amide linkages. Therefore, by this method, α-chymotrypsin can be immobilized covalently via glutaraldehyde and PEI to the LB membrane.
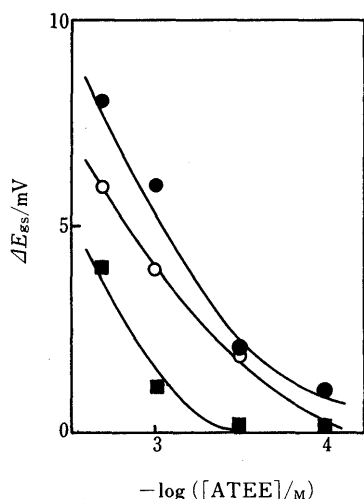
Fig. 6. The Calibration Graphs of the Sensors Prepared with Active Ester LB Membranes

The contents of active ester in the LB membrane were 10 (—●—), 40 (—○—), and 90% (—■—).
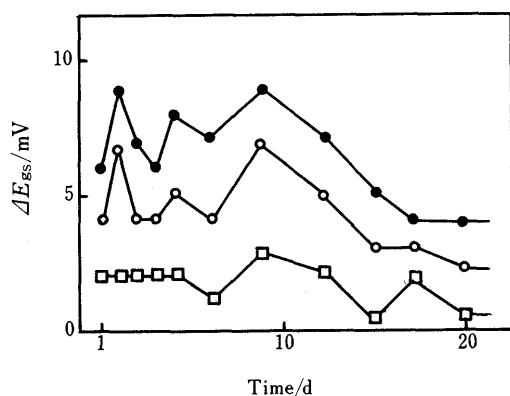

Fig. 7. The Long-Term Stability of the Sensor Prepared with 90% Active Ester LB Membrane

The ΔE_{gs} values were measured for 2 (—●—), 1 (—○—), and 0.3 mM (—□—) ATEE in 2 mM phosphate buffer at pH 8.0.

Figure 6 shows typical calibration graphs of an ISFET sensor modified with α-chymotrypsin by this method. The response was enhanced with increasing content of active ester in the LB layer. This tendency is consistent with the fact that PEI is bound to the LB membrane covalently not by adsorption. The long-term stability of the sensor is shown in Fig. 7. However, the long-term stability of this sensor could not improved compared with that of the above-mentioned sensor.

Thus, it has been shown that PEI can be used as a reactive spacer to immobilize α-chymotrypsin on the surface of LB membranes. However, there still remained problems such as immobilizing a sufficient amount of the enzyme and the long-term stability for sensor applications, although insertion of PEI as a spacer did improve the properties of the sensor. Further improvement of the PEI method and the immobilization of emzymes other than α-chymotripsin are now under study.

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