0273-2289/93/4101-2—0041$03.00

Enzyme Modification of LB Membrane-Deposited ISFET

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INTRODUCTION

The ISFET enzyme sensors can be prepared by modification of the gate surface with enzymes that catalyze the reaction consuming or producing an H^+ ion. Based on this idea, many kinds of ISFET enzyme sensors have been developed so far (*1–6*). In general, enzymes can be immobilized to the gate surface of ISFET by the use of organic thin membranes. The organic membranes where enzyme is immobilized should be a crucially important factor in determining the performance of the sensors, because the signal, usually H^+ ion, is produced as a result of an enzyme-catalyzed reaction of analyte on and/or in the membrane. Additionally, the enzyme activity and the diffusion rates of analytes and reaction products should depend on the chemical and physical properties of the membrane. Bearing these in mind, we have examined the performance of ISFET enzyme sensors with LB membrane gates. Several techniques are described for immobilizing enzymes on the surface of LB enzymes.

IMMOBILIZATION OF ENZYME ON THE SURFACE OF LB MEMBRANES

It has been demonstrated that the LB membrane coated on the ISFET provides a barrier, to some extent, for the H^+ ion to transport from bulk solution to the gate surface (*7*). However, the LB membrane cannot block the penetration of the ion completely. These results suggest that the use of LB membrane-coated ISFET makes possible preparation of enzyme sensors, by immobilization of enzyme on the surface of the LB membrane.

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This report was presented at the US/Japan Workshop on Microfabrication and Biosensors, July 21–24, 1992, sponsored by the National Science Foundation.

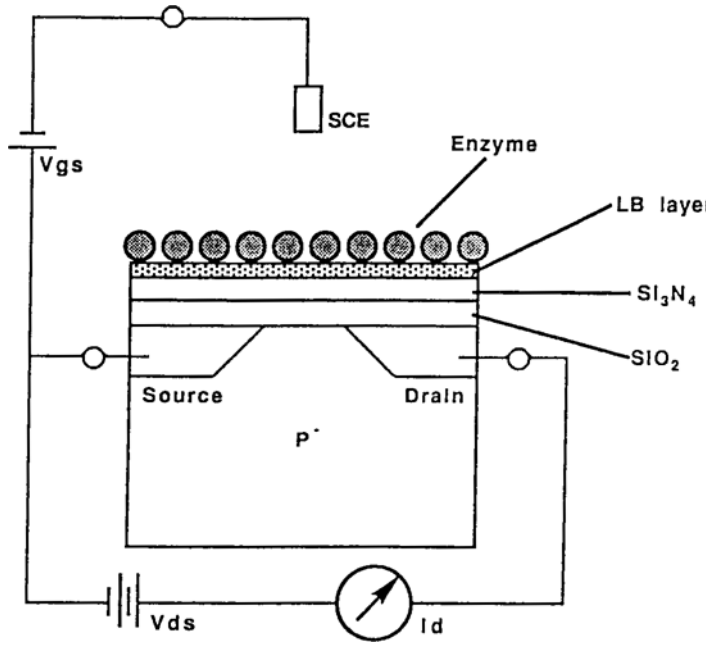
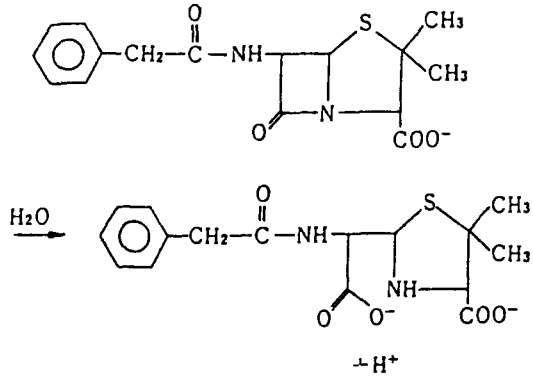


Fig. 1. Schematic illustration of LB membrane-coated ISFET enzyme sensor.

Adsorption Method

We have prepared a penicillin G sensor by this technique using stearic acid LB membrane (*8*). The procedure for the preparation of the sensor is as follows: A stearic acid multilayer was deposited on Si_3N_4 gate ISFET. After drying the LB layer on the ISFET, the probe was immersed in ca. 0.5% penicillinase solution at 0–5°C for 15 h to immobilize the enzyme on the LB membrane. The penicillin G sensor was rinsed thoroughly with the working buffer before use. The structure of the sensor was schematically shown in Fig. 1. The potentiometric response of the penicillin G sensor is based on the pH change resulting from the enzymatic reaction (1) on the surface of the LB membrane.



(1)

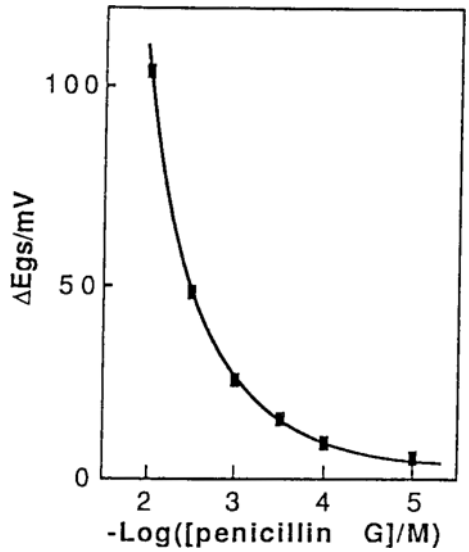


Fig. 2. Potentiometric response of the LB membrane-coated ISFET penicillin sensor.

The potentiometric response of the sensor for penicillin G was tested in a 5 m*M* buffer at pH 8.0 (Fig. 2). For 0.1–10 m*M* penicillin G solutions, the gate potential always shifted in the positive direction to the extent depending on the concentration of penicillin G, and reached steady-state values in 4–5 min. This shows that penicillinase was adsorbed to the stearic acid LB membrane without deactivation. The response time of the sensor was not so rapid as to be expected from the fact that the thickness of the LB layer was 500 Å or less. The reproducibility of the response was satisfactory. It is clear that the LB membrane and penicillinase remain on the gate surface without denaturation, even after repeated use.

The long-term stability of the sensor was checked by measuring the output voltage for 3 m*M* and 10 m*M* penicillin G. The probe was stored in buffer at 4°C when not in use. Fig. 3 shows that the sensor is fairly stable under these experimental conditions. This sensor could be stored also in dry state (e.g., in air) for a long time without any loss of the enzyme activity, which is convenient for practical use of the sensor.

Polymer Spacer Method

It is not always possible to immobilize an enzyme on the LB membrane by electrostatic or hydrophobic force of attraction. For this reason, reactive LB membranes that can bind enzymes via covalent linkage have been developed for biosensor applications (*9*).

We have used a reactive polymer as a spacer that can link the LB membrane and enzyme (Fig. 4) (*10*). The device was prepared in the following manner. A Y-type LB membrane composed of stearic acid was deposited on the ISFET gate so as to protrude the carboxyl groups to the uppermost

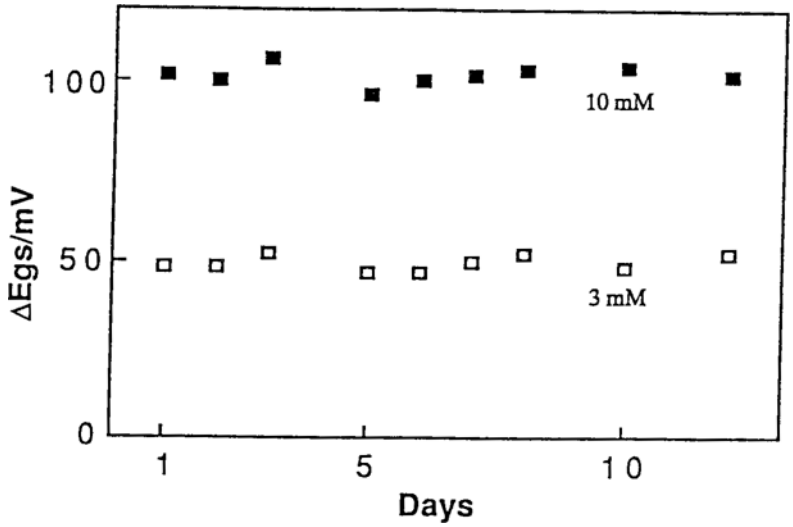


Fig. 3. Long-term stability of the penicillin sensor.

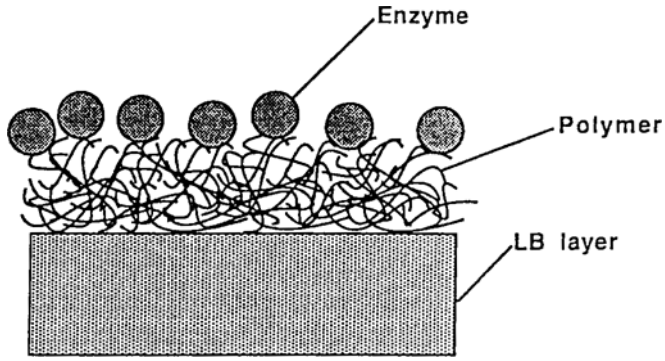
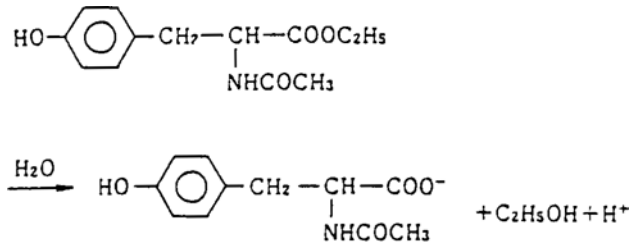


Fig. 4. Schematic representation of the LB membrane surface bearing polyethyleneimine and enzyme.

surface. The LB membrane-coated ISFET was immersed in 0.3% polyethyleneimine (PEI) solution (pH 7) for 2 h, by which PEI could be adsorbed to the LB membrane through electrostatic interaction. After being treated with 3% glutaraldehyde, the probe was dipped in a 0.5% enzyme solution to bind the enzyme. A typical response and the long-term stability of the ISFET protein sensor prepared by the use of α-chymotrypsin are shown in Figs. 5 and 6. The probe exhibited a potentiometric response to *N*-acetyltyrosine ethyl ester (ATEE) (a model substrate of protein for α-chymotrypsin), showing that the enzyme was immobilized withoug deactivation and catalyzed the hydrolysis reaction of ATEE as follows:



(2)

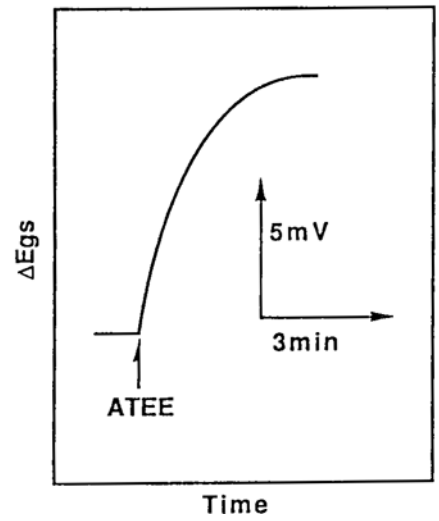


Fig. 5. Typical response of chymotrypsin-immobilized ISFET to ATEE. Sample: 2 m*M* ATEE in 2 m*M* buffer at pH 8.

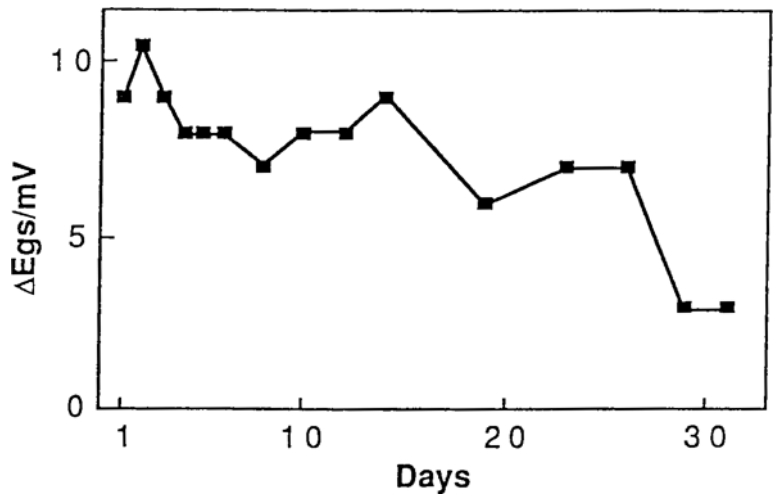
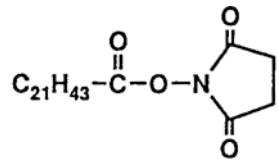
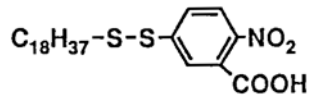


Fig. 6. Long-term stability of the chymotrypsin-immobilized ISFET. The gate potential was measured to 2 m*M* ATEE in 2 m*M* buffer at pH 8.



Succinimidyl behenoate 1



5-(Octadecyldithio)-2-nitrobenzoic acid 2

Fig. 7. Reactive amphiphiles for LB membrane preparation.

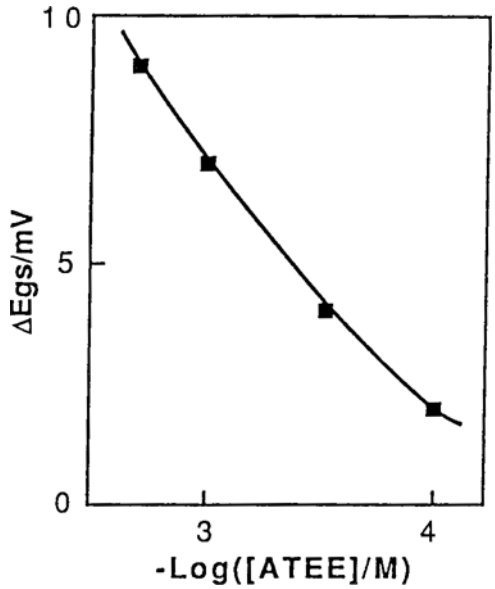


Fig. 8. Typical calibration graph of the ATEE sensor prepared with ISFET coating the active ester LB membrane.

Active Ester and Disulfide Methods

An alternative method to immobilize the enzyme covalently on the surface of the LB membrane is to use reactive amphiphiles as membrane-forming material. We have prepared two kinds of reactive amphiphiles: succinimidyl behenoate 1 (*11*) and 5-octadecyldithio-2-nitrobenzoic acid 2 (*12*) (Fig. 7). The mixed LB membrane of 1 and stearyl alcohol could be deposited on the ISFET gate with deposition ratio of ca. 1. The mixed LB membrane-coated ISFET was treated with a 0.5% α-chymotrypsin solution to bind the enzyme through amide linkage, by the reaction of active ester with amino residues in enzyme. Fig. 8 shows a typical response of

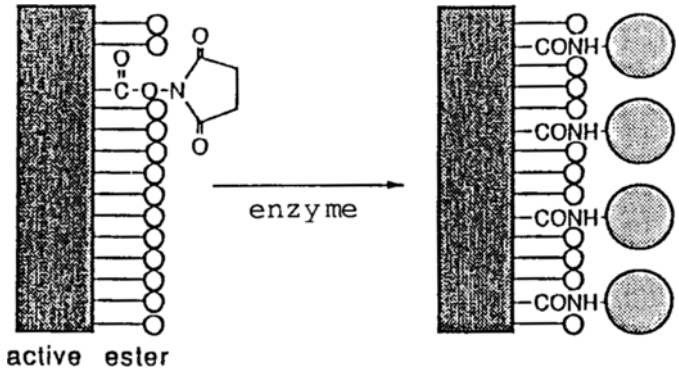


Fig. 9. Immobilization of enzyme on the active ester LB membrane 1.

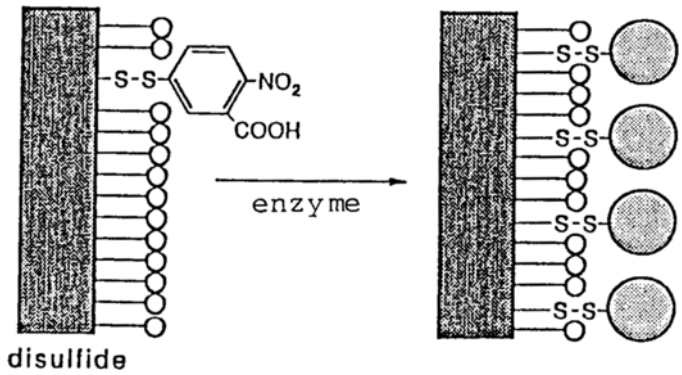


Fig. 10. Immobilization of enzyme on the disulfide LB membrane 2.

the α-chymotrypsin-modified ISFET that was prepared by the use of the mixed LB membrane containing 90% active ester. The sensor showed a response to ATEE over a concentration range of 0.1–2.0 m*M*. It is rationally understood that amino residues in the enzyme attacked the active esters in the LB layer to form an amide likage between them. The reaction between the enzyme and the active ester in the LB layer is schematically illustrated in Fig. 9.

The LB membrane 2 was also deposited on the ISFET gate, and further modified with urease through a thiol/disulfide exchange reaction (Fig. 10) (*12*). In order to immobilize urease, the ISFET modified with the LB membrane 2 was simply immersed in the enzyme solution at T_r for 1 h. The sensor thus prepared was sensitive to urea over the concentration range of 0.3–30 m*M*, which is comparable to those of the urea sensors based on a urease-albumin crosslinked membrane (*13*). However, the long-term stability of the sensor was not satisfactory. The reaction between the enzyme and the disulfide LB membrane is schematically shown in Fig. 10.

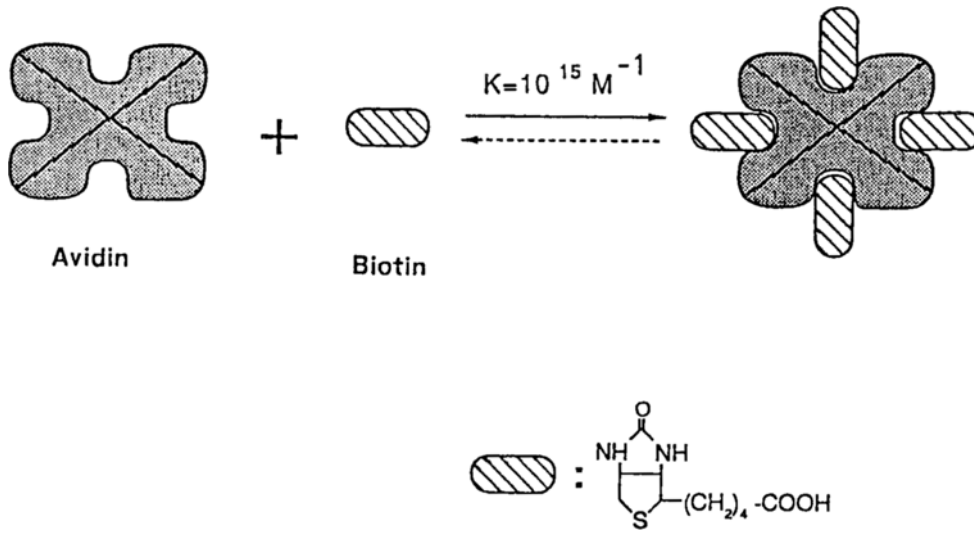


Fig. 11. Avidin-biotin system.

Avidin-Biotin Method

The simple adsorption method is often too fragile to obtain reliable enzyme sensors. On the other hand, covalent immobilization is frequently encountered with the deactivation of enzymes, probably because of the undesirable conformational changes. Judging from these situations, to obtain stable and highly active enzyme membranes enzymes should be immobilized noncovalently but tightly. This idea prompted us to use a binding protein to immobilize the enzyme on the LB membrane surface. We employed avidin-biotin system for this purpose (Fig. 11). It is well known that avidin is a glycoprotein that has an exceptionally strong affinity to biotin (binding constant: $10^{15}M^{-1}$) (*14*). Additionally, avidin is generally adsorbed to the surface of hydrophobic materials. Based on these characteristics of the avidin-biotin system, biotinylated glucose oxidase (GOx) was immobilized through complexation with avidin, which was adsorbed to the hydrophobic LB membrane. In this case, the ITO electrode was used to prepare an amperometric glucose sensor, in place of ISFET device.

Typical calibration graphs are shown in Fig. 12. It is clear that the avidin-biotin system is effective to immobilize GOx on the surface of the LB membrane. We have employed two different techniques to immobilize GOx by the avidin-biotin system. In the first technique, avidin is adsorbed to the LB membrane surface through hydrophobic interaction and then the biotinylated GOx is immobilized by complexation with the avidin (bridge method). The other technique includes the direct adsorption of the complex between avidin and biotinylated GOx prepared in advance in solution (ABC method). The ABC method gave a higher response than

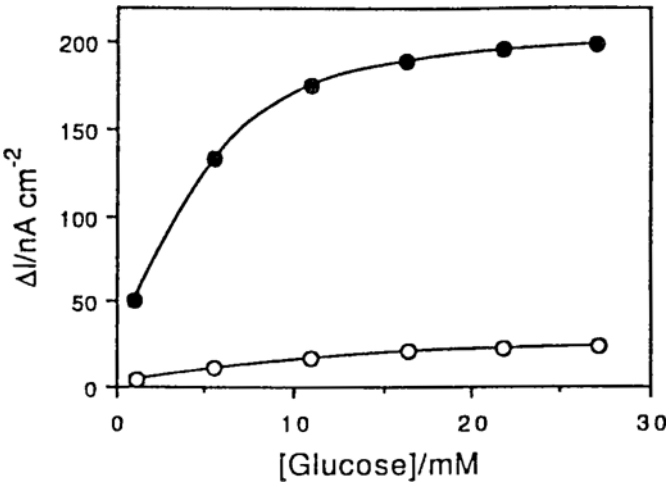


Fig. 12. Typical calibration curves of the glucose sensor prepared by two methods of the avidin-biotin system. Bridge method (○) and ABC method (●).

the bridge method, which may be because of the difference in enzyme loading between the two methods.

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