Reactive Langmuir-Blodgett Membrane for Biosensor Applications. Use of Succinimidyl Behenoate-Based Membranes as Support for Covalently Immobilizing α-Chymotrypsin

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Synopsis. Succinimidyl behenoate was synthesized to prepare Langmuir-Blodgett (LB) membranes which possess reactive groups on the surface. The surface of the succinimidyl behenoate-based LB membrane could be covalently modified with α-chymotrypsin, for the purpose of biosensor applications.

Several groups have reported the use of Langmuir-Blodgett (LB) membranes for preparing enzyme sensors.[1-5] A prerequisite to construction of LB membrane-based enzyme sensors is to immobilize enzymes on or in the LB membrane deposited previously, or to deposit enzyme-containing monolayer using, for example, Fromherz-type trough. We have prepared an enzyme sensor by immobilizing penicillinase through adsorption on the surface of LB membrane deposited on a pH-sensitive field effect transistor (pH-ISFET). Penicillinase, fortunately, could be tightly adsorbed, through electrostatic or hydrophobic force of attraction, to the surface of the LB membrane. However, it is not always possible for enzymes to be adsorbed to the LB membrane surface without deactivation. In fact, we have observed poor reproducibility in potentiometric response for the pH-ISFETs covered with LB membranes adsorbing urease, trypsin, and α-chymotrypsin, presumably due to desorption or deactivation of enzymes. For these reasons, we have developed a reactive LB membrane which can covalently bind enzymes on the surface, using a fatty acid active ester, succinimidyl behenoate (**1**), as a membrane-forming amphiphile.

This paper reports the immobilization of α-chymotrypsin on the surface of LB membrane containing **1** which is deposited on the pH-ISFET and its use as an enzyme sensor.

Experimental

Materials. Succinimidyl behenoate (**1**) was prepared from *N*-hydroxysuccinimide (1.2 g) and behenic acid (3.4 g) in 50 ml chloroform using dicyclohexylcarbodiimide (2.1 g) as coupling reagent. The crude product was purified by repeated recrystallization from ethanol. 3.2 g (73%). Mp 98—99 °C. Found; C, 71.46; H, 10.59; N, 3.36%. Calcd for $C_{26}H_{47}NO_4$; C, 71.35; H, 10.82; N, 3.20%.

α-Chymotrypsin (EC 3.4.21.1) was obtained from Sigma Co. *N*-Acetyltyrosine ethyl ester (ATEE) was purchased from Research Organics Inc.

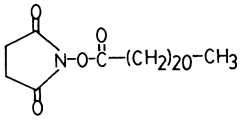


Chart 1. Succinimidyl behenoate **1**.

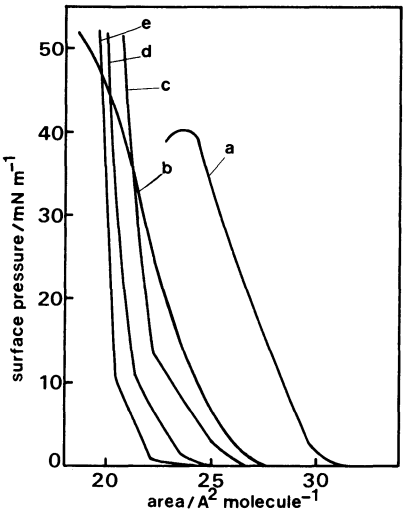


Fig. 1. π-A isotherms of **1** and **1**/1-octadecanol mixed monolayers on water at 17 °C. The contents of **1** in the monolayers were 100(a), 90(b), 40(c), 10(d), and 0 w/w%(e).

The procedure for the fabrication and the pH response of the pH-ISFET used are described elsewhere.[6,7]

Sensor Fabrication. The **1** or **1**/1-octadecanol mixed monolayer was spread with benzene on an aqueous subphase in a Langmuir trough (Kyowa Kaimenkagaku Co.). The water subphase was adjusted at ca. pH 7 with $BaCl_2$ and $KHCO_3$. The monolayer was deposited on the pH-ISFET precoated with 11-layer octadecanoic acid LB membrane, by dipping the probe vertically through the monolayer at 25 mN m^{-1}. After the monolayer had been removed from water surface, the probe was pulled up. By this treatment, the active ester groups should be located at the uppermost surface of the deposited LB layer, and, therefore, the surface is reactive to nucleophilic group such as amine. The deposition behavior of **1** and **1**/1-octadecanol membranes was elucidated by the deposition on the glass plate precoated with octadecanoic acid LB layer (1 cm×3 cm), since the small area of the pH-ISFET (ca. 1 mm×3 mm) inhibited precise measurement of deposition ratio.

The LB membrane-coated pH-ISFET, thus prepared, was immersed in a 0.5% α-chymotrypsin solution (1 mM phosphate buffer, pH 7.5, 1 M=1 mol dm^{-3}) for 4 h, in order to bind the enzyme onto the surface of LB membrane. The enzyme sensors prepared were rinsed thoroughly with the working buffer before use.

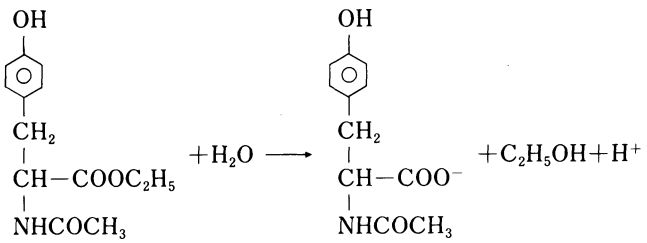
Results and Discussion

In Fig. 1, we display π-A diagrams of **1** monolayer together with the mixed monolayers of **1** and 1-octadecanol spread on water surface at 17 °C. Al-

though the **1** monolayer can be compressed up to about 40 mN m^{-1}, the π–A diagram does not afford clear indication of the formation of condensed monolayer of **1**. Basically the same π–A diagram as **1** was observed for the mixed monolayer with 90 w/w% **1**. These monolayers may assume expanded phase, not condensed one. On the other hand, the mixed monolayers with lower **1** content (10 and 40%) exhibited π–A diagrams with transition pressures at 11 — 14 mN m^{-1}, which are similar to that of 1-octadecanol monolayer, indicative of the formation of stable condensed phase.

We examined the deposition property of the monolayer on the glass plate precoated with octadecanoic acid LB layers. The deposition ratios of the **1** and mixed monolayers were measured by dipping the glass plate into the water subphase through the monolayer at 25 mN m^{-1}. The deposition ratios thus measured were 1.9 for **1** monolayer and 1.3, 1.0, and 1.1 for the mixed monolayers with **1** content of 90, 40, and 10%, respectively. Unexpectedly high values of deposition ratios for the monolayers of higher **1** content might come from the expanded nature of the **1** and 90% **1** monolayers on the water surface at 25 mN m^{-1}.

Figure 2 shows a typical response of the α-chymotrypsin-immobilized pH-ISFET which was prepared by the use of the probe covered with the mixed LB layer (**1** content; 90 w/w%) as well as a pH response of bared pH-ISFET. The potentiometric response of immobilized pH-ISFET became slow to compare with that of bared pH-ISFET. However, the probe exhibited a potentiometric response to *N*-acetyltyrosine ethyl ester (ATEE) (a model substrate of α-chymotrypsin), showing that the enzyme was immobilized without deactivation and catalyzed hydrolysis reaction of ATEE to change pH value at the surface of the pH-ISFET gate as follows:



ATEE

It is thought that amino residues in the enzyme attacked the active ester of LB layer to form amide linkage between them, in view of the fact that succinimidyl esters are widely used for modifying proteins[8] and covalently binding proteins on the solid support.[9] The potentiometric response of the sensor was fairly reproducible.

We also tried to immobilize α-chymotrypsin on the surface of 1-octadecanol LB layer deposited on the pH-ISFET, by immersing the probe in the enzyme solution (0.5%) in a similar manner. After rinsing the probe with the working buffer for ca. 1 h, we measured the potentiometric response of the sensor to 2 mM ATEE repeatedly and found that the ΔE_{gs} value

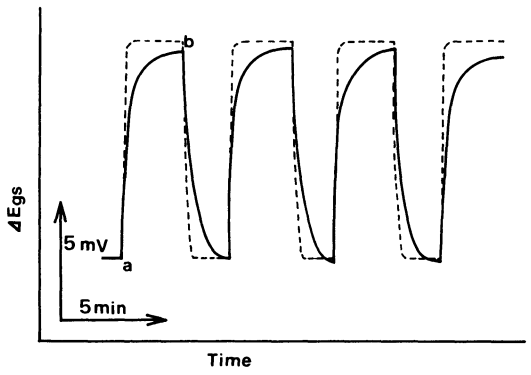


Fig. 2. A typical response of the α-chymotrypsin-immobilized ISFET for *N*-acetyltyrosine ethyl ester. The probe was immersed in the ATEE solution (2 mM) (a) and buffer solution (b). 2 mM buffer (pH 8.0) was used. High concentration samples (above 2 mM) of ATEE could not be prepared due to the low solubility. A response of bared pH-ISFET from pH 8.0 buffer to pH 7.8 buffer is shown in dashed line (---).

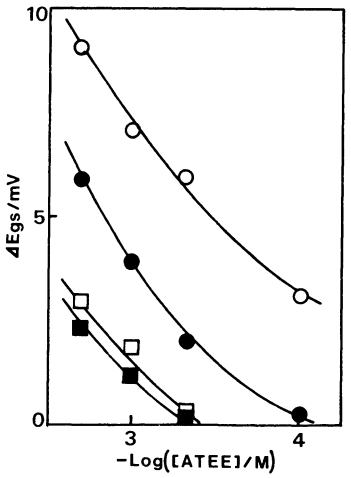


Fig. 3. Effects of **1** content in the LB membrane on the response of the sensor. The contents of **1** were 90(—○—), 40(—●—), 10(—□—), and 0 w/w%(—■—). 2 mM buffer (pH 8.0) was used. High concentration samples (above 2 mM) of ATEE could not be prepared due to the low solubility.

reduced gradually during the repeated use; ΔE_{gs}= ca. 2 mV after 4 measurements (data not shown). This clearly shows the essential role of the active ester to bind enzyme effectively.

The effects of **1** content in the mixed LB membrane on the response of the α-chymotrypsin-immobilized pH-ISFET were examined (Fig. 3). The potentiometric response was enhanced with increasing the **1** content in the LB membrane. This may be due to enchanced enzyme loading on the surface of higher **1** content. A small portion of enzyme seems to be adsorbed to the surface without formation of amide linkage, judging from the fact that the sensor prepared by the use of 1-octadecanol LB membrane showed a little response. The pure **1** LB layer was omitted from

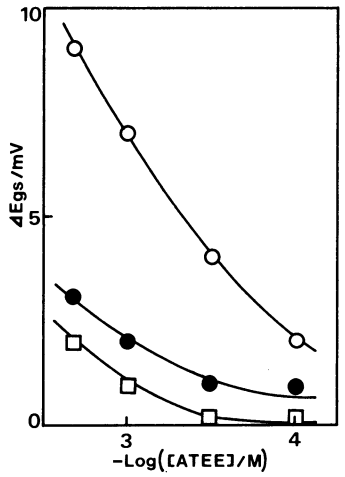


Fig. 4. Effects of pH of the sample solution on the response of the sensor. The pH values of the solutions were 8.0(—○—), 7.0(—●—), and 6.0(—□—).

the consideration, because the nonordered surface structure in deposited **1** layer was suggested by the anomalously high value of deposition ratio.

Figure 4 shows the response of the sensor prepared by the use of 90% **1** mixed LB membrane in pH 6, 7, and 8. The potentiometric response was higher in pH 8 medium, but, in pH 6 and 7 media, the response was lower. This may be due to the fact that the optimum pH of α-chymotrypsin is pH 8.0.[10]

It is a problem in the present method that the long-term stability of α-chymotrypsin immobilized is not satisfactory, which seems to depend on the conditions of measurement and storage. For instance, the catalytic activity of the sensor disappeared in a few days even if the sensor was stored in a 2 mM phosphate buffer (pH 8.0) at 4 °C. However, the long-term stability may be enhanced by the more suitable design of the LB membrane surface and the improvement of the storage conditions.

Thus, we have shown that succinimidyl behenoate is a useful material for the preparation of reactive LB membrane which can bind enzyme covalently on the surface, for the biosensor applications.

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