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Enzyme Electrode for Hydrogen Peroxide Using a New Enzyme-Immobilization "Polymer-Enzyme Aggregate"

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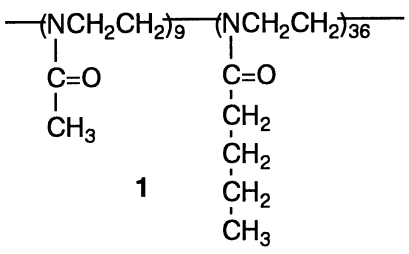
An enzyme electrode for hydrogen peroxide was developed with the aid of an easy and effective enzyme-immobilization. The enzyme electrode was made by holding an aggregate of a self-assembled amphiphilic block copolymer **1** and catalase on an oxygen electrode by use of microporous acetylcellulose membranes. The response of the electrode was linear between 4 X 10^{-6} - 4 X 10^{-4} M of hydrogen peroxide and remained unchanged after 50 days with over 150 measurements.

Enzyme electrodes have been studied for the past two decades and many research efforts have been devoted to them.[1,2] An important factor in enzyme electrodes is the immobilization of enzymes on proper electrochemical devices, such as gas, pH and ion-selective electrodes, as enzyme membranes. Enzymes are usually immobilized by covalent binding to proper supports (or to electrode itself) or by gel entrapment with poly(acrylamide) etc. However, these immobilization methods need complicated and time-consuming procedures. Few studies have so far been done from the viewpoint of "easiness" in making enzyme membranes. We have found that an amphiphilic block copolymer **1** forms an aggregate, which can entrap various compounds including enzymes, in aqueous media.[3,4] The aggregate easily encapsulates enzymes only by mixing **1** and enzymes in a phosphate buffer solution at room temperature.

In this paper we report an application of the polymer-enzyme aggregate, in which the aggregate (**1**-catalase aggregate) is sandwiched between two porous polymeric membranes to provide an enzyme membrane for hydrogen peroxide electrode. Several enzyme electrodes which have involved conventional immobilization methods mentioned above for hydrogen peroxide have already been reported.[5-10] The



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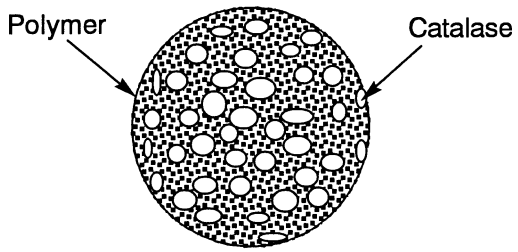

Polymer-catalase aggregate

polymer-enzyme aggregate electrode competes with these conventional electrodes in terms of sensitivity and durability and excels them in terms of easiness in making enzyme membranes.

The copolymer **1** consisting of poly[*N*-(acetylimino)ethylene] (hydrophilic chain) and poly[*N*-(pentanoylimino)ethylene] (hydrophobic chain) was prepared by two-step block copolymerization between 2-methyl-2-oxazoline and 2-butyl-2-oxazoline according to a literature.[11] The number average molecular weight of **1** was estimated to be 4,200 by the vapor pressure osmometer measurement. The unit ratio of **1** was determined to be 9:36 by ^{1}H-NMR. Catalase from bobin liver (EC 1.11.1.6) was obtained from Sigma Chemical Co. Catalase catalyzes a reaction: $2H_2O_2 \rightarrow 2H_2O + O_2$. Aggregate of **1** and catalase was prepared by mixing **1** (300 mg) and the enzyme (20 mg) in a 0.05 M phosphate buffer solution (pH 7.0, 10 mL) with a stirring at 23±2 °C for overnight to give a clear homogeneous solution. The free polymer and catalase were removed by a dialysis. It was confirmed from UV-VIS spectrophotometry (407 nm) that almost all of catalase initially charged was encapsulated into the aggregate. The dynamic light scattering study of an aqueous phosphate buffer solution of the aggregate using a COULTER N4SD indicated that the average diameter of the aggregate was ca. 0.13 μm.

An enzyme assay was carried out as follows. An aqueous solution (0.2 mL) containing the aggregate (0.15 mg or 0.015 mg as enzyme) or free catalase (0.15 mg or 0.015 mg) was added to 5.8 mL of a 0.05 M phosphate buffer solution (pH 7.0) which contained ca. 24 mM hydrogen peroxide and the reaction mixture was incubated at 30 °C. The consumption of hydrogen peroxide was determined spectrophotometrically by the reaction with titanium sulfate according to a literature.[12]

A polymer-enzyme aggregate membrane was prepared as follows. An aliquot of the aggregate solution (0.48 mL) in which the catalase concentration was 2.1 mg/mL was filtered through a porous acetylcellulose membrane (MF-Millipore filter, 25 mm diameter) with suction. Another acetylcellulose membrane was attached, resulting in the immobilization of the aggregate, in which ca. 1.0 mg of catalase was present, between the two membranes.

The aggregate membrane was placed on the tip of an oxygen electrode (Model GU-S, Iijima Denshikogyo, Gamagoori, Aichi) in a biosensor system with thermostated flow cell (30 °C) (NA-SO11, Nakano Vinegar Co., Ltd., Handa, Aichi). Distilled water was transferred into the flow cell at a rate of 4 mL/min. After the current output of the electrode reached a steady state, sample solutions which contained hydrogen peroxide and 0.05 M phosphate buffer (pH 7.0) were injected into the flow cell for 2.5 min. The current output of the oxygen electrode increased and reached a steady state a few minutes after injecting the sample. The difference in the current output between the initial and steady state stages was measured. The enzyme membrane was stored in a 0.05 M phosphate buffer solution (pH 7.0) at 4 °C between measurements.

Figure 1 shows the time course of consumption of hydrogen peroxide at 30 °C in the enzyme assay. The catalytic activity of free enzyme is somewhat higher than that of the polymer-enzyme aggregate which contains the same amount of enzyme. However, the enzyme within the aggregate can adequately maintain its catalytic activity.

Current differences for the polymer-enzyme aggregate electrode (log ΔI) are plotted against the concentration of hydrogen peroxide (log [H_2O_2]) (Fig. 2). The response of the electrode decreases as the pore size of acetylcellulose membrane is varied 0.05 μm > 0.1 μm > 0.025 μm > 0.22 μm > 0.45 μm. It is noted that the 0.05 μm membrane gives the highest response. As previously mentioned, average diameter of

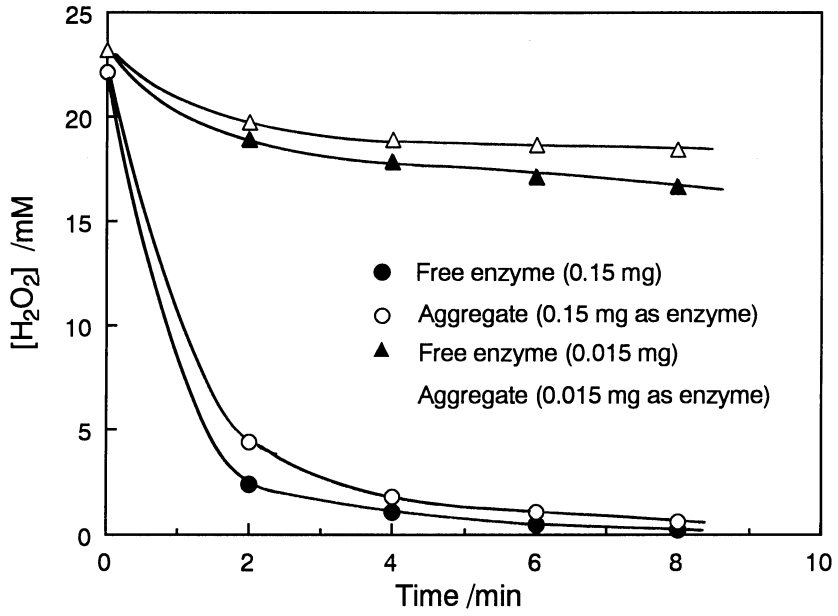

Fig. 1. Time course of the consumption of H_2O_2 at 30 ℃ in enzyme assay.

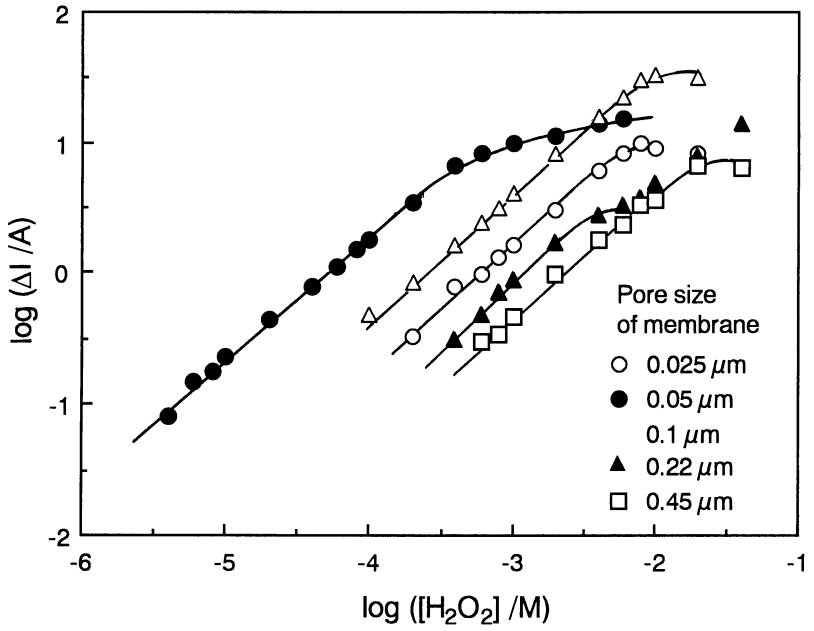

Fig. 2. Calibration graph for the polymer-enzyme aggregate electrode.

the polymer-enzyme aggregate is ca. 0.13 μm. It is anticipated that the aggregate is the most effectively entrapped by the 0.05 μm membrane. The membranes which have larger pore sizes cannot hold the aggregate well while in case of the 0.025 μm membrane material diffusion through the membrane pore does not favorably work. Linear parts of the calibration graphs (Fig. 2) are 4×10^{-6} - 4×10^{-4} M of hydrogen peroxide for the 0.05 μm membrane and 1×10^{-4} - 1×10^{-2} M for the 0.1 μm membrane.

When a suspension of 1 mg of free catalase was placed between two acetylcellulose membranes, the electrode response was extremely low and decreased as the pore size of the membrane was varied 0.025 μm > 0.05 μm > 0.1 μm. Namely, the free enzyme is too small in size to be sufficiently entrapped by the membranes.

The polymer-enzyme aggregate electrode with the 0.05 μm membrane almost kept its initial response after 50 days. More than 150 measurements were carried out during this time.

To our knowledge, it is anticipated that the present method in which polymer-enzyme aggregates are sandwiched between two porous polymeric membranes is the easiest preparation of enzyme membrane. Such a easy preparation would be applied not only to biosensors but also to other biocatalyst devices.

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