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Application of polymaleimidostyrene as a convenient immobilization reagent of enzyme in biosensor

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Abstract

Sulfhydryl groups of glucose oxidase (GOD) were reacted with maleimide groups of polymaleimidostyrene (PMS) which was coated onto the porous carbon sheet, and the carbon sheet immobilized by GOD was combined with an oxygen electrode to fabricate a glucose sensor. The activity of thiolated GOD immobilized to PMS is much larger than that of native GOD immobilized to PMS. The good linear relationship of glucose and oxygen current response was obtained in a concentration range from 0.1 to 2 mM and upper limit of linear range was found to be 3.0 mM. The immobilized GOD activity is highly dependent on pH at immobilization and the maximum activity was obtained at pH 5.5, probably because the SH groups of GOD that are indispensable for generation of enzyme activity is not exposed at this pH. It was found that PMS is very effective reagent to immobilize enzyme strongly via covalent bond, because high density of maleimide groups of PMS can catch not only exposed SH groups but also buried SH groups.

Keywords: Polymaleimidestyrene; Glucose sensor; Thiolation of enzyme; Conformation change

1. Introduction

There have been a number of immobilization methods of enzymes for fabricating biosensors [1,2]. In these methods, the stable immobilization can be realized by formation of covalent bond between enzyme and functional groups of carrier. The immobilization of enzyme on electrode surfaces can be classified into four categories, that is, adsorption, gel entrapment, covalent coupling and crosslinking. Chemically reactive sites of a protein may be amino groups, carboxyl groups, phenol residues of tyrosine, sulfhydryl groups or the imidazole group of histidine and in most cases amino and carboxyl groups of enzyme have been used for stable immobilizations. Sulfhydryl moeties of enzymes have also been used to immobilize to maleimide bound surface [3]. However, very few studies have been reported on immobilization of enzyme using sulfydryl group of enzyme in biosensor fabrication [4,5], although the reaction of sulfydryl group and maleimide group has been widely used to conjugate enzyme to antibody [6–8] in enzyme linked immunosorbent assay (ELISA). The reason why sulfhydryl group has not been used for immobilization is that the stable introduction of a number of maleimide groups to the surface of carrier is not easy, compared with those of amino and carboxyl groups.

In 1991, polymaleimidostyrene (PMS) was synthesized by one of us [9] and this polymer has a plenty of maleimide groups on polystyrene chain and adsorbs strongly to porous carbon material. If it can be coated onto the porous carbon material, enzymes can be easily immobilized to gas permeable porous material by only an immersion of PMS coated porous material into enzyme solution.

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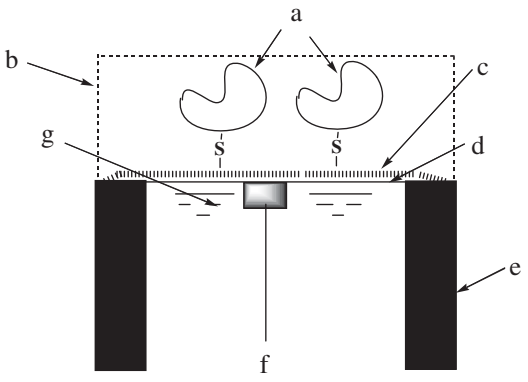

Fig. 1. A cross sectional view of the glucose sensor fabricated by combining oxygen electrode and GOD immobilized PMS coated porous carbon sheet. a: enzyme, b: dialysis membrane, c: porous carbon sheet, d: oxygen permeable membrane, e: electrode body, f: gold electrode, g: electrolyte of oxygen electrode.

In this research, PMS was used to immobilize glucose oxidase (GOD), and the porous carbon sheet immobilized GOD was combined with an oxygen electrode. It is well known that maleimide group reacts with sulfhydryl group very rapidly and has possibility that reacts with amino group. The reactivity of amino group of amino acid and *N*-ethylmaleimide (NEM) was investigated in previous works [10–12], but the reaction product was not identified. On the other hand, it is well known that the reaction rate of maleimide group and thiol compound is very fast [13,14], and hydrolysis of maleimide proceeds slowly [15]. In this study, in order to estimate the reaction of enzyme and maleimide groups of PMS, the reaction rate of lysine and NEM has been studied and it was compared with that of cysteine and NEM, and the formation of adduct of lysine and NEM was confirmed by GC MS spectrophotometry.

The use of PMS has an effective merit that PMS can be adsorbed to a variety of hydrophobic materials such as polystylene, polyethylene and polyetheretherketone (PEEK) which have been difficult to use as immobilization carriers for biosensors, because the introduction of functional groups to the surface of these hydrophobic materials are not so easy to be carried out. Enzymes have many sulfydryl groups in their bodies, and activity should be decreased if many sulfydryl groups react with PMS because the conformation of native enzyme must be damaged.

In this work glucose oxidase from *Aspergillus niger* was used to illustrate the usefulness of PMS for enzyme immobilization. This enzyme consists of two subunits. The sequence of the *Aspergillus niger* glucose oxidase gene has been elucidated [16,17], one subunit has three cysteine residues and 15 lysine residues. The catalytic properties of GOD from *Aspergillus niger* has been reported [18–20] and this enzyme is not interfered with SH reagent such as mercury [21]. It can be expected that the reactivity of GOD to PMS is improved by thiolation of GOD, then we also examined thiolated GOD as well as native GOD.

2. Experimental

2.1. Reagents and chemicals

The reagents used in this experiment were reagent grade chemicals. GOD from *Aspergillus niger* was purchased from Wako Pure Chemical Industries Ltd. In order to purify the enzyme, GOD solution was dialyzed against distilled water and lyophilized.

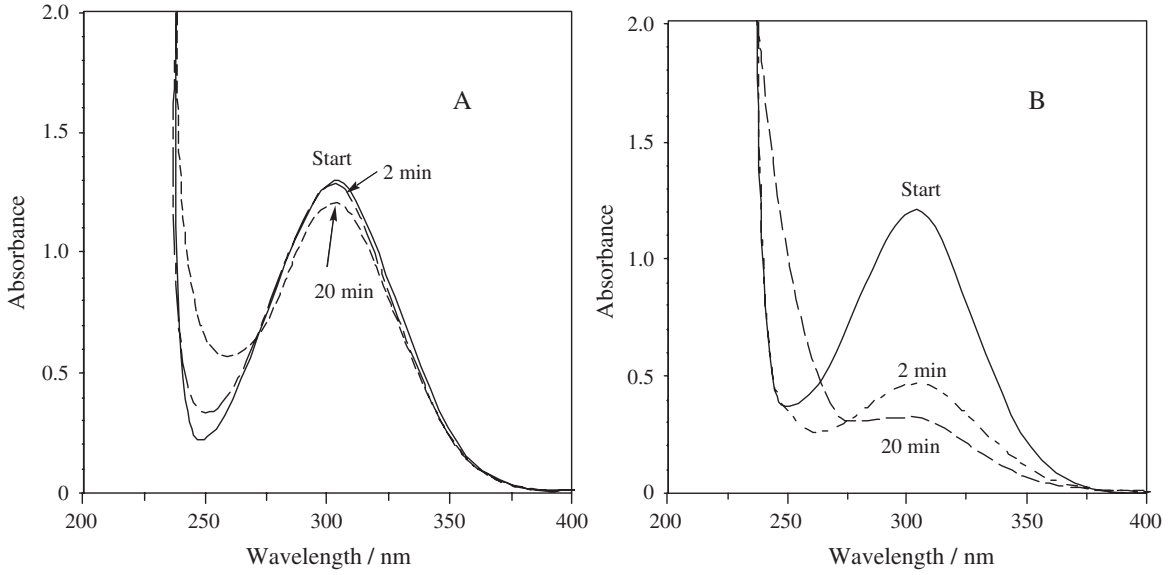

Fig. 2. Absorption spectra of hydrolysis of NEM (A) and a mixture of NEM and lysine (B) at each reaction time. pH 9.5 Initial concentrations of NEM and lysine are 2.0 mM. reaction time; a: 0 min, b: 2 min, c: 20 min.

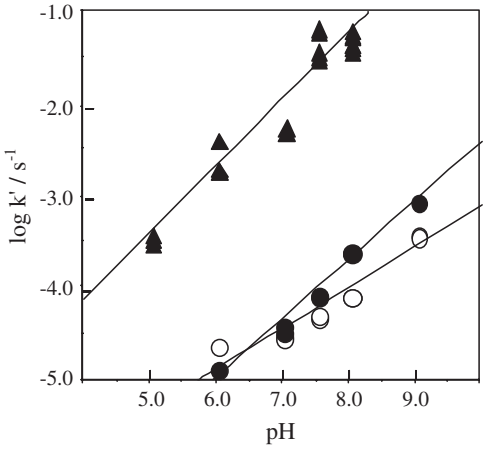

Fig. 3. The pH dependencies of logarithms of pseudo first order rate constants of each reaction. Initial concentration; EMI=1.0 mM, cysteine=10 mM, lysine=10 mM. ▲: NEM and cysteine, ● NEM and lysine, ○ hydrolysis of NEM.

Porous carbon sheet (Kansai Tar Ltd., 90% voids) used for immobilization carrier of enzyme consists of 0.1 μm carbon fibers and its thickness is 2 mm.

2.2. Thiolation of GOD

GOD modified with thiol moieties was obtained by the reaction of enzyme and *s*-acetylmercaptosuccinic anhydride (SAMSA). Thiol modification was performed according to following procedure. First, SAMSA standard solution (10 mg/ml) was prepared by dissolving SAMSA into 0.01 M phosphate buffer (pH 5.5). Next, 30 μl of this standard solution was added to 1 ml of 1 mg/ml GOD solution, and incubated to introduce thiol groups to lysine residues of GOD for 15 minutes at room temperature.

2.3. Spectrophotometry and GCMS

In order to estimate the reaction rate constants of *N*-ethylmaleimide (NEM) and cysteine or lysine, the time dependencies of the absorbance of NEM caused by these reactions were measured using spectrophotometer (JASCO CRT-400). The pH dependencies of the reaction rates were obtained. The buffer solution was adjusted by 0.1 M NaH_2PO_4 and 0.1 M NaOH. Amino acids (lysine, cysteine) and NEM used were reagent grade chemicals. The reaction products of lysine and NEM, and hydrolyzed product of NEM were identified by a GC-MS spectrometer (JEOL, JMS-SX102).

2.4. Amperometry

A potentiostat (NPOT 2501) and one pen recorder were used to carry out the typical amperometric experiment. Dissolved oxygen was decreased accompanied by GOD reaction of glucose. An oxygen electrode (TOA DKK) was used to monitor an oxygen concentration consumed by GOD reaction.

Polymaleimidostyrene (PMS) was supplied by Tsukuba Materials Information Laboratory (Japan), and 0.1 mg/ml of PMS solution was prepared by dissolving PMS into chloroform solution. A porous carbon sheet (5 mm diameter, 0.2 mm thickness) was immersed into this PMS solution for 30 min and PMS adsorbed carbon paper was dried.

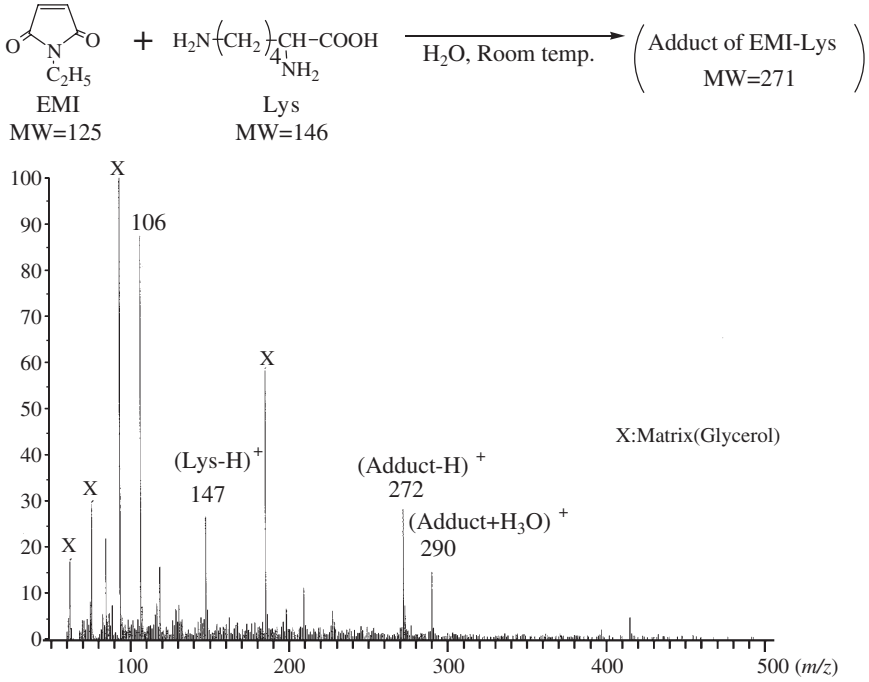

Fig. 4. Mass spectrum of the reaction products of lysine and NEM initial condition :0.1 M lysine and 0.01 M NEM, pH 9.5, reaction time is 60 min.

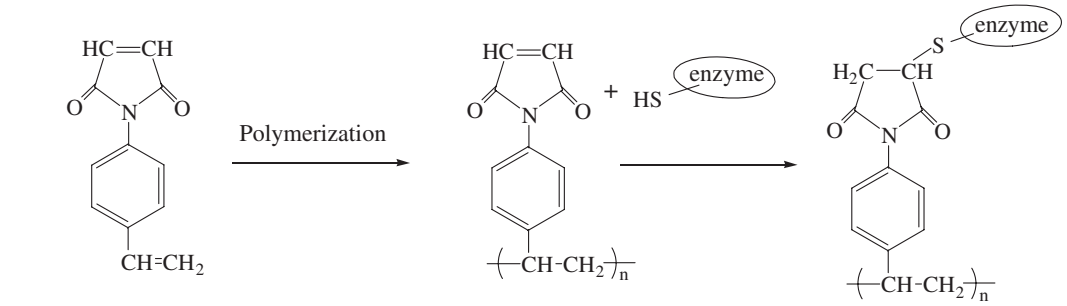

Fig. 5. Illustration of immobilization reaction of enzyme to polymaleimidostyrene.

After immobilization, adsorbed enzyme was removed by washing using 0.1 M phosphate buffer, and carbon sheet coated with GOD immobilized PMS was prepared. This GOD modified carbon sheet was placed on an oxygen membrane of oxygen electrode and covered with a dialysis membrane, then, glucose sensor was fabricated. The combinations of an oxygen electrode or an ammonia electrode with the enzyme immobilized porous carbon materials in biosensor fabrication have been reported in previous works [22–24]. The cross-sectional view of the present glucose sensor is shown in Fig. 1.

The response characteristics of the glucose sensor such as response time and sensitivity were investigated. In order to prevent from the further reaction of excess maleimide groups with immobilized GOD, a reminder of free maleimide groups of PMS were blocked by reacting with 0.01 M aminoethanethiol for 10 min.

The immobilization of GOD and amperometry were carried out at room temperature throughout all the experiments.

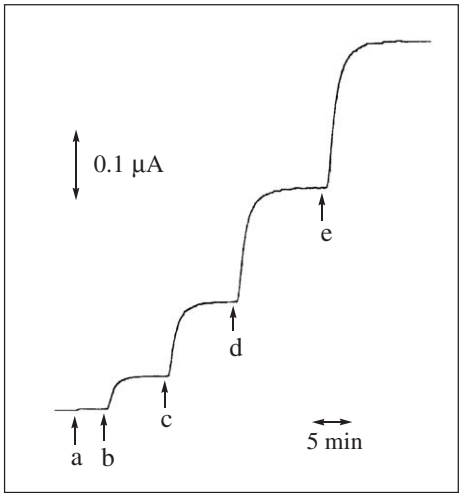

Fig. 6. Glucose response curves obtained by an oxygen electrode equipped with thiolated GOD immobilized PMS coated carbon sheet treated with aminoethanethiol. pH 5.5. Added amount of glucose; a: 0.02 mM, b: 0.2 mM, 0.4 mM, d: 0.6 mM, e: 0.8 mM.

3. Results and discussion

The double bond of maleimide group undergoes an alkylation reaction (Michael-type electrophilic addition) by forming a stable thioether bond with sulfydryl groups. The reaction rate has been shown to be several orders of magnitude faster than that between the maleimide group with amino group. NEM absorbs ultraviolet ray at 300 nm, and we found that this absorption disappears by addition of not only sulfydryl group but also amino group to maleimide group. Therefore, the reaction rates of NEM and cysteine or lysine were investigated. The absorbances of NEM obtained at each reaction time of NEM and lysine are shown in Fig. 2. From these results, the pseudo-first order reaction rate constant of amino group of lysine and maleimide group was found to be 0.003 s^{-1} at pH 9.5. The data in Fig. 2 were obtained by unbuffered lysine solution (pH 9.5). The pH dependences of reaction rates of NEM and cysteine, lysine and hydrolysis were studied by using phosphate buffer and are shown in Fig. 3.

The hydrolysis of NEM takes place at alkaline media, as well as the reaction of NEM and lysine, but the decrease rate of absorbance of NEM by lysine is

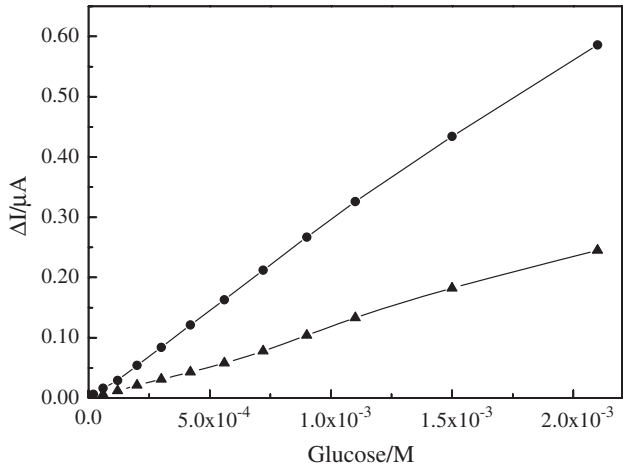

Fig. 7. Calibration curves of an oxygen electrode GOD immobilized PMS coated carbon sheet. pH 5.5. ▲: native GOD, ●: thiolated GOD.

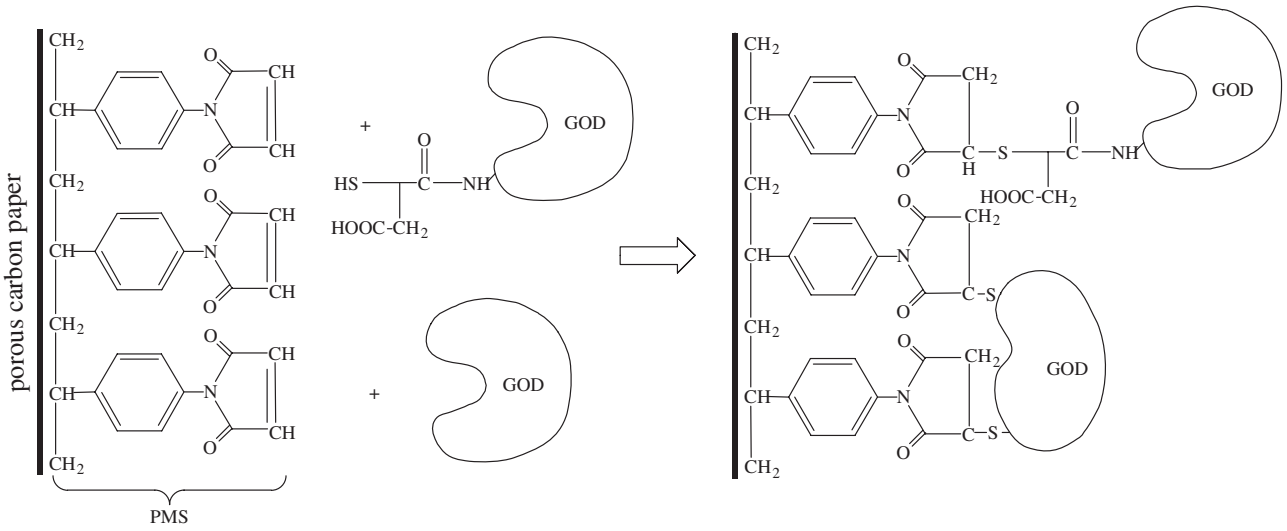

Fig. 8. Illustration of immobilization types of GOD.

significantly larger than that by hydrolysis, indicating that lysine reacts with maleimide group slowly. The reaction rates of NEM and amino acids decreased as pH decreased, and the plots of logarithm of pseudo-first order rate constant (k′) and pH showed a linear relationship and this reaction was found to be the first order with respect to proton concentration ($k' = k[H^+]$, k represents the second order rate constant). No absorbance decrease was observed at pH 5.5 after 30 min of reaction of 1 mM NEM and 10 mM lysine. Then, it can be recognized that the reaction of lysine and maleimide does not occur in acidic media, and it can be concluded that the immobilization of enzyme by the reaction of lysine residue and maleimide does not take place at below pH 7.

The reaction products of lysine and maleimide group in aqueous solution have not been reported until now, then, we identified the reaction products by GC-MS. The mass spectrophotometric data of the reaction products of lysine and NEM is shown in Fig. 4. This data indicates that not only lysine bonded NEM (m/e=271) but also hydrolyzed products of NEM are produced. From the data of ^{1}H NMR of reaction products, the adduct of lysine to NEM was found to be α-*N*′-(5-carboxy-5-amino)penthylamino-*N*-ethyl succinimide.

On the other hand, sulfydryl group can react with maleimide group very rapidly in a broad pH range, although its rate constant decreases as pH decreases. The rate constant of cysteine and NEM at pH 5.5 was estimated to be approximately 0.001 s^{-1} which is so rapid that the cysteine of enzyme can be reacted with maleimide group. Then if enzyme is immobilized to PMS, its immobilization must be occurred by the reaction of maleimide group and sulfhydryl group. Fig. 5 shows the illustration of immobilization of enzyme to PMS.

Fig. 6 shows the response curves of glucose obtained by an oxygen electrode equipped with native GOD immobilized PMS coated carbon sheet, and this result indicates that this sensor exhibits an excellent response for glucose and the response time (95% of the steady sensing signal) is about 12 min. However, an oxygen electrode equipped with PMS uncoated carbon sheet immersed into GOD solution showed no glucose response after washing.

An oxygen electrode equipped with thiolated GOD immobilized carbon sheet showed glucose response about 2.5 times as large as that equipped with native GOD immobilized carbon sheet. The calibration curves of glucose obtained by both electrodes are shown in Fig. 7. The good linear relationship of glucose and oxygen current response was obtained in a concentration range from 0.1 to 2 mM and upper limit of linear range was found to be 3.0 mM. This result indicates that the activity of immobilized thiolated GOD is higher than that of immobilized native GOD if the immobilized amount of thiolated GOD is equal to that of native GOD , because the number of bonding sites of the native GOD to PMS must be larger than that of

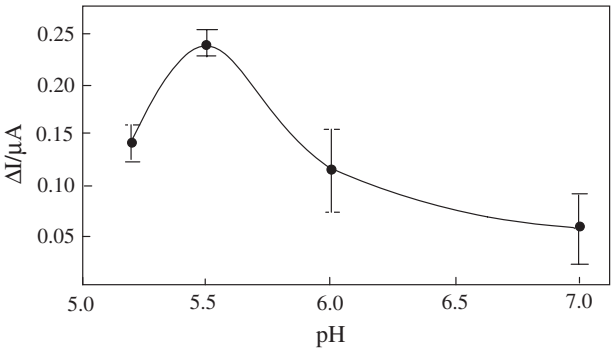

Fig. 9. The effect of pH at immobilization of GOD to PMS on the immobilized GOD activity glucose concentration: 2.2 mM. ●:mean values of five data.

the immobilized thiolated GOD to PMS, resulting in severe damage of conformation of GOD. PMS has a lot of maleimide groups which tends to react with buried SH groups of GOD, but thiolated GOD reacts with modified sulfhydryl group which is exposed on the surface of GOD, and the other buried sulfhydryl groups are hard to be reacted with maleimide groups. In order to confirm this speculation, the measurement of the amount of immobilized GOD to PMS is needed to be carried out. Fig. 8 shows the speculative illustration of immobilization of enzymes to PMS.

The effect of pH at immobilization of GOD to PMS on the immobilized GOD activity is shown in Fig. 9, and this result shows that the immobilized enzyme activity is significantly influenced by pH of the reaction of sulfhydryl group and maleimide group. However, the reaction rate of sulfhydryl group of free cysteine monomer and maleimide monomer is not significantly changed in this pH range. This fact suggests that conformation of enzyme may be highly influenced by pH change. The optimum pH of free GOD is known to be 5.6, at which the maximum activity of immobilized GOD is obtained.

On the other hand, the activity of the immobilized thiolated GOD to PMS is not changed at pH change from 5 to 7, and this fact indicates that the exposed SH group introduced by thiolation must be reacted with PMS.

In order to clarify the effect of maleimide to free GOD activity, free GOD solution was confined on the oxygen gas membrane with a dialysis membrane, and the glucose response was studied by this free enzyme confined oxygen electrode As a result, it was found that glucose response did not decrease by adding maleimide monomer at pH 7.0, and this fact indicates that SH groups indispensable for generation of activity in free GOD do not react with maleimide monomer, although it reacted with maleimide groups located on the polystyrene chain of PMS.

The sensor response using thiolated GOD immobilized PMS was decreased as much as 40% after 5 days, and this value was improved to be 20% by reacting remained maleimide groups with aminoethanthiol. This fact indicates that unreacted sulfhydryl groups of immobilized GOD gradually react with maleimide groups of polystyrene chain. From the results described above, it can be recognized that PMS is an excellent polymer reagent for immobilization of enzyme conveniently, because PMS may have a strong affinity to hydrophobic polymer material such as polystyrene or polyetheretherketone which has not been used for immobilization carrier of proteins. PMS is also attractive one which can study structure change of enzyme because highly condensed maleimide groups of polystyrene chain can catch SH groups of conformation changed enzyme. Then it can be expected that PMS would be widely used in biosensor technology in the near future.

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