Grafting of Polyacrylamide onto Silica Gel and Its Application to Enzyme Immobilization

Masato SHIMOMURA*, Masaki ASAKURA*, Nan WANG,* Takeshi YAMAUCHI* and Shinnosuke MIYAUCHI*

Polymerization of acrylamide was carried out in a redox system consisting of ceric ion and mercapto groups introduced onto silica gel. In the course of the polymerization, polyacrylamide was grafted onto the surface of the silica gel. The amount of the grafted polymer reached 130 mg per gram of the silica gel employed in the polymerization. In order to immobilize glucose oxidase on the silica gel, the coupling reaction between the grafted polymer and the enzyme was attempted in the presence of a condensing agent. It was shown that 4.3 mg of the enzyme was immobilized on 1 g of the polyacrylamide-grafted silica gel, and the enzyme-immobilized product had an apparent activity of 50 units/g. The pH-activity and temperature-activity profiles of the immobilized enzyme resembled those of the native enzyme. A kinetic study of the glucose oxidation with the immobilized enzyme allowed the consideration that the enzyme immobilization caused retardation of lactone formation from the enzyme-glucose complex. The immobilized enzyme kept 95% of its original activity in water over a period of 3 months.

Key words: Polyacrylamide / Silica Gel / Enzyme / Glucose Oxidase / Immobilization

1. Introduction

Inorganic materials have been modified by grafting of organic polymer chains onto their surfaces[1)-5)]. The inorganic materials modified by the grafting technique have surface properties derived from the polymer chains grafted and, therefore, can be subjected to further organic modification by applying the functional groups of grafted polymer to incorporation of a variety of organic molecules.

Enzymes have been fitted for a variety of practical purposes by immobilization on insoluble supports[6)-12)]. In immobilizing enzymes on inorganic supports, the grafting technique is useful because a large number of functional groups of grafted polymer chains can be applied to covalent immobilization. Polymerization of a vinyl monomer, for example, in a redox system consisting of ceric ion and reducing groups introduced onto inorganic solid particles by silane coupling has been attempted, and it has been shown that polymer chains can be grafted onto the particles[3)]. If the grafted polymer has functional side-groups which react with enzyme molecules to form covalent linkages, they can be employed in immobilization of the enzyme on the particles.

Received May 19, 1997

*Department of BioEngineering, Nagaoka University of Technology

In this paper are reported the results of grafting of polyacrylamide (polyAAm) onto silica gel and immobilization of glucose oxidase (GOD) on the polyAAm-grafted silica gel. PolyAAm was grafted onto silica gel through redox polymerization of acrylamide (AAm) initiated by the reaction between ceric ion and mercapto groups introduced onto silica gel, and GOD was immobilized on the polyAAm-grafted silica gel in the presence of a condensing agent. Activity of the immobilized GOD was measured under various conditions.

2. Experimental

2. 1 Materials

The silica gel used in this work was Wakogel Q-63 obtained from Wako Pure Chemical Ind., Ltd., which had a particle size below 325 mesh and a BET surface area of 847 m^2/g. The GOD used (EC 1.1.3.4, grade II, from *Aspergillus* sp.) was supplied by Toyobo Co., Ltd, which had an activity of 156 units/mg. The peroxidase (POD) used (EC 1.11.1.7, type I, from horseradish) was supplied by Sigma Chemical Co., which had an activity of 116 units/mg. AAm obtained from Wako Pure Chemical Ind., Ltd. was recrystallized from benzene and sublimed under reduced pressure prior to use. 3-Mercaptopropyltrimethoxysilane (MPS) from Kanto Chemical Co., Inc. was used without further purification. Other chemicals were guaranteed-reagent grade or analytical

grade commercial materials and used without further purification.

2．2 Grafting of polyAAm onto silica gel

Grafting of polyAAm was carried out by following the scheme shown Fig.1. The silica gel was treated with MPS to introduce mercapto groups onto its surface as described in a previous publication[3]. Grafting of polyAAm by the redox polymerization was conducted as follows: Into a flask, 0.5 g of the silica gel treated with MPS, 3.0 g of AAm and 10.0 ml of distilled water was charged. After deaeration of the mixture, a solution of 0.2 mmol of ceric ammonium nitrate in 3.0 ml of 1 *N* nitric acid was added. The polymerization was carried out at 30℃ with stirring under nitrogen. After a given time, the reaction mixture was poured into a large excess of acetone to precipitate the polymer and silica gel. The precipitate was dried at a temperature below 60℃ *in vacuo*. The conversion was determined by the following equation:

Conversion (%)
=100{Precipitate(g)-Silica gel(g)}/AAm used(g)

In order to isolate polyAAm-grafted silica gel, the precipitated product was dispersed in distilled water and centrifuged at 10^5m/sec^2 until the silica gel was precipitated completely. The precipitated silica gel was dispersed in distilled water and centrifuged once more. This procedure was repeated several times, and the precipitated silica gel was dried at a temperature below 60 ℃ *in vacuo*.

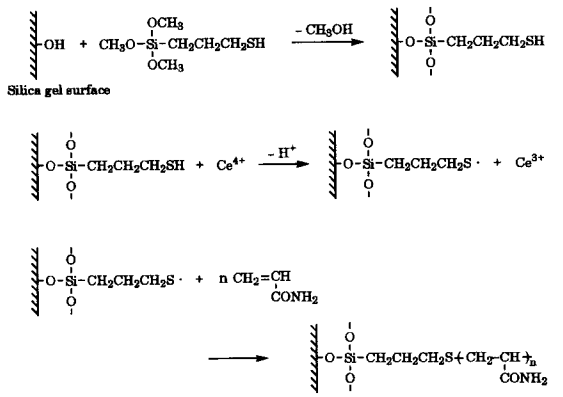

Fig. 1 Grafting of polyAAm onto silica gel surface.

The polyAAm-grafted silica gel thus isolated was analyzed by means of IR spectroscopy. IR spectra were recorded on a JEOL JIR-7000 spectrometer.

2．3 Immobilization of GOD on silica gel

Immobilization of GOD on the polyAAm-grafted silica gel was carried out by use of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene-sulfonate (CMC) as a condensing agent[7].

A mixture of 0.5 g of the polyAAm-grafted silica gel, 17 mg of GOD and 7.0 ml of 0.1*M* phosphate buffer (pH 6.5) was placed into a flask and stirred for 5 min. Subsequently 80 mg of CMC was added and stirring was continued. After 18 h of stirring, the reaction mixture was centrifuged at 10^5m/sec^2, and the silica gel was precipitated completely. The precipitated silica gel, *i.e.* GOD-bound silica gel, was dispersed in distilled water, filtered off, and washed on filter with distilled water. This washing procedure was repeated several times. The GOD-immobilizing reaction and the succeeding washing were conducted at 4℃.

2．4 Quantitative analysis of immobilized GOD

The amount of immobilized GOD was estimated by using the Folin-Ciocalteu phenol reagent after alkaline copper treatment, according to the direction of Lowry[13].

A 50 ml of 2.0% solution of Na_2CO_3 in 0.1 *N* NaOH solution and 1.0 ml of 0.5% solution of $CuSO_4 \cdot 5H_2O$ in 1.0% sodium tartrate solution was mixed, and 2.5ml of the mixture was added to a suspension of 5.0 mg of GOD-bound silica gel in 0.5 ml of distilled water. The suspension was stirred and allowed to stand for 10 min at room temperature. To the suspension 0.25 ml of the Folin-Ciocalteu phenol reagent diluted to 1 *N* in acid was added very rapidly and mixed within a second or two. After 30 min or longer, the silica gel particles in the suspension were filtered off, and the filtrate was subjected to colorimetry. Absorbance at 660 nm was measured on a Shimadzu UV-3100 PC spectrometer. The

amount of immobilized GOD was calculated from a standard curve obtained with solutions of 10–250 μg of native GOD in 0.5ml of distilled water.

2．5 Measurement of GOD activity

The activity of immobilized GOD was measured by the colorimetric method based on the procedure of Trinder[14]. The method includes the reaction of hydrogen peroxide, produced in oxidation of glucose by GOD, with phenol and 4-aminoantipyrine in the presence of POD to yield a colored product. The amount of the colored product is regarded as the measure of the GOD activity.

In a dark bottle, 32.0mg of 4-aminoantipyrine and POD were dissolved in 200 ml of 0.1 *M* phosphate buffer, and a solution of 4.2mg of phenol in 4.0ml of distilled water was added : *Solution 1*. *D*-Glucose solution of 1.0m*M* was prepared and allowed to stand for 12 h or more at room temperature : *Solution 2*. Avoiding direct sunlight, 5.0 ml of *Solution 1* and 0.5 ml of *Solution 2* were kept at a given temperature and mixed with 1.0 mg of GOD-bound silica gel, and the mixture was incubated at the given temperature. After 60 min the mixture was cooled to 0℃ and incubated for 5 min. Then the silica gel particles were filtered off rapidly, and the filtrate was subjected to measurement of absorbance at 505 nm. The activity of immobilized GOD was calculated from a standard curve obtained with 1–25 μg of native GOD. The activity was measured over the pH range 5–9 and over the temperature range 20–60℃.

The colorimetric method was applied also to measurements of the GOD reaction rate at various glucose concentrations. Obtained data were employed to discuss kinetic effect of the immobilization.

3. Results and discussion

3．1 Grafting of polyAAm onto silica gel by redox polymerization

Fig.2 shows the result of polymerization of AAm in the redox system consisting of ceric ion and mercapto groups introduced onto silica gel. By the polymerization for 15 h, *ca*. 20% of AAm was converted into polyAAm, and 10% of the polymer was grafted onto the silica gel. The amount of the grafted polymer was 130mg per gram of the silica gel employed in the polymerization. IR spectrum of the polyAAm-grafted silica gel is shown together with that of untreated silica gel in Fig.3. In the IR spectrum of the polyAAm-grafted silica gel, absorptions characteristic

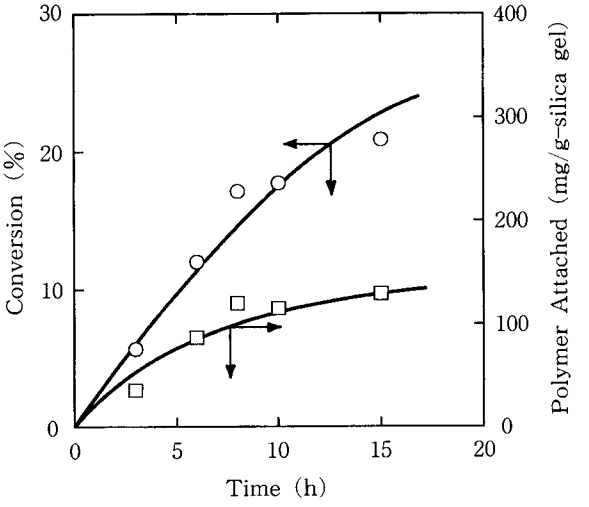

Fig. 2 Polymerization of AAm in the redox system consisting of ceric ion and mercapto groups introduced onto silica gel.

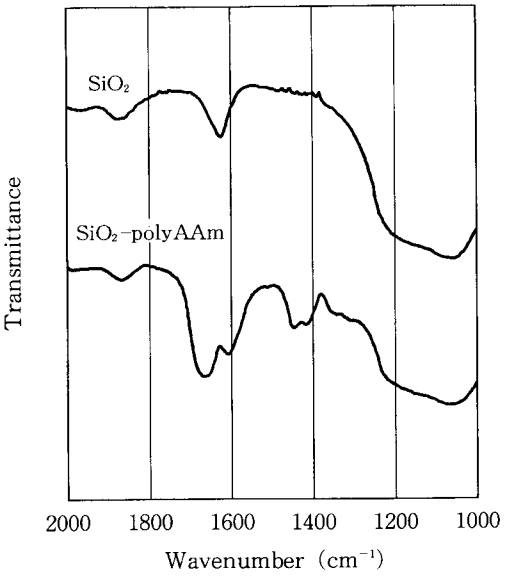

Fig. 3 IR spectra of untreated silica gel (SiO_2) and polyAAm-grafted silica gel (SiO_2-polyAAm).

of polyAAm were observed clearly at 1670 cm^{-1} due to stretching of C=O bond in amide group and near 1450 cm^{-1} due to CH_2 deformation.

In the presence of untreated silica gel which had no mercapto groups on its surface, on the other hand, the polymerization of AAm was considerably slow, giving the result that at most 5% of AAm was converted to polymer in 24 h, and no polymer was grafted onto the silica gel.

3. 2 Immobilization of GOD on polyAAm-grafted silica gel

As shown in Table 1, GOD was immobilized successfully on the poly-AAmgrafted silica gel by use of CMC as a condensing agent, while no GOD was immobilized in the absence of CMC. The GOD-immobilized product from the poly-AAm-grafted silica gel prepared by use of CMC had a high apparent activity of 50 units/g, though the specific activity of the immobilized GOD was reduced to 7.5% of the native one's. The amount of GOD immobilized on untreated silica gel was, in spite of the use of CMC, significantly small as compared with the case of polyAAm-grafted silica gel. Considering that the specific activity of the immobilized GOD was relatively high, the immobilization on untreated silica gel may be due to adsorption of GOD on the silica gel.

Table 1 Amount and activity (at 30℃, pH 7.0) of immobilized GOD.

Support	Condensing agent	GOD immobilized (mg/g-support)	Apparent activity (units/g-product)	Specific activity (units/g-GOD immobilized)
Untreated silica gel[a]	CMC	0.2	19.5	97.5
Grafted silica gel[b]	—	0	0	—
Grafted silica gel[b]	CMC	4.3	50.3	11.7

a No polyAAm was grafted.
b The amount of grafted polyAAm was 130 mg/g-silica gel.

It is known that amide nitrogen attacks carboxylic carbon nucleophilically to give imide linkage, *i. e.* -CO-NH-CO-[15]. Similar reaction can occur between the amide groups of polyAAm and the carboxyl groups of GOD. Therefore, it is likely that, in the presence of CMC, GOD was covalently immobilized on the polyAAm-grafted silica gel by the condensation reaction between the amide groups of grafted polyAAm and the carboxyl groups of GOD.

3. 3 Effect of pH and temperature on activity of immobilized GOD

Fig.4 shows the effect of pH on the activity of native GOD and the GOD immobilized on polyAAm-grafted silica gel. It is obvious that the

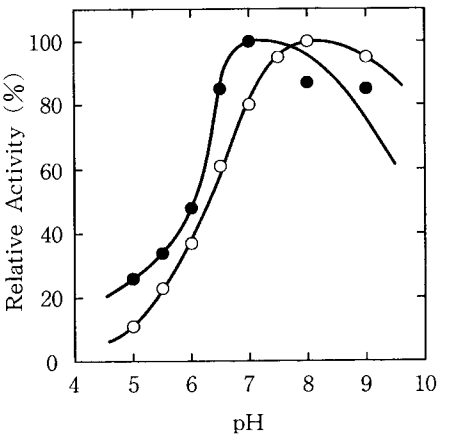

Fig. 4 Effect of pH on the activity (at 30 ℃) of GOD : ○, native ; ●, immobilized on polyAAm-grafted silica gel.

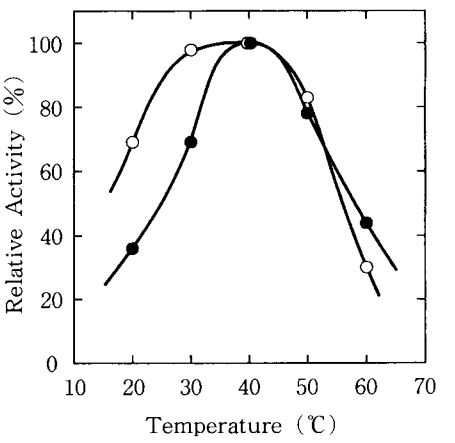

Fig. 5 Effect of temperature on the activity (at pH 7.0) of GOD : ○, native ; ●, immobilized on polyAAm-grafted silica gel.

optimum pH for the immobilized GOD was between 7.0 and 8.0, which was similar to the case of the native GOD. In addition, the overall pH-activity profiles of the native and immobilized GOD resemble each other. Fig.5 shows the effect of temperature on the activity, in which it is seen that the optimum temperature for both the native and immobilized GOD was at around 40℃. The temperature-activity profiles of the native and immobilized GOD also resemble each other. These results suggest that the nature of GOD of responding to such environmental conditions as pH and temperature was not affected remarkably by the immobilization.

3. 4 Kinetic effect of immobilization

Assuming that the glucose oxidation with GOD proceeds through Michaelis-Menten mechanism, the reaction is described as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

where, E, S, ES and P represent the enzyme (GOD), the substrate (β- *D* -glucose), the enzyme-substrate complex and the product (*D* -glucono- δ -lactone), respectively. The rate constants are given by k_1, k_2 and k_3. If the steady state where the concentration [ES] is constant, *i. e.*, k_1 [E][S] is equal to (k_2+k_3) [ES], is assumed, the glucose oxidation rate V $(=k_3$ [ES]) is given by

$$V=V_{max}[S]/(k_m+[S])$$
$$V_{max}=k_3[E]_0$$
$$k_m=(k_2+k_3)/k_1$$

where $[E]_0$ represents a initial concentration of the enzyme, which is equal to [E]+[ES]. V_{max} and K_m are called the maximum reaction rate and Michaelis constant, respectively. The reciprocal of the rate V is presented by

$$1/V = (K_m/V_{max})/[S] + 1/V_{max}$$

which means that the plots of 1/V against 1/[S](Lineweaver-Burk plots) give a straight line, and the intercepts on the 1/V axis and 1/[S] axis give the values of $1/V_{max}$ and $-1/K_m$, respectively.

In order to study the kinetic effect of the immobilization, the rates of glucose oxidation reaction with native GOD and the GOD immobilized on polyAAm-grafted silica gel were measured at various glucose concentrations. Fig.6 shows Lineweaver-Burk plots for the glucose oxidation with the native and immobilized GOD. The plots gave straight lines which were of typical Michaelis-Menten form.

The maximum reaction rates V_{max} and the apparent Michaelis constants K_m determined from Fig.6 are presented in Table 2. The V_{max} value for the immobilized GOD was smaller than that for the native GOD. The difference in V_{max} between the native and immobilized GOD is considered to be due to difference in the rate constant k_3, which suggests that glucose oxidation with the immobilized GOD resulted in retardation of lactone formation from the GOD-glucose complex. The decrease of K_m value by the immobilization corresponds to decrease of the rate constant k_3.

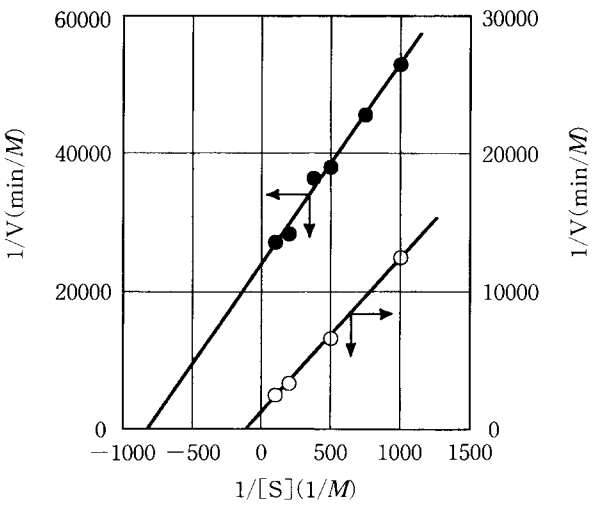

Fig. 6 Lineweaver-Burk plots for the glucose oxidation (at 30℃, pH 7.0) with GOD : ○, native; ●, immobilized on polyAAm-grafted silica gel. The concentration of GOD was 10mg/l.

Table 2 Kinetic parameters for native and immobilized GOD.

GOD	V_{max}(m*M*/min)	K_m(m*M*)
Native	0.85	9.5
Immobilized	0.04	1.2

3. 5 Stability of immobilized GOD

The stability of the GOD immobilized on polyAAm-grafted silica gel was examined in water (pH 7.0). A mixture of 0.5 g of the GOD-bound silica gel and 50 ml of distilled water was stored at 4℃ in the dark, and the activity (at 30℃, pH 7.0) of the immobilized GOD was measured periodically.

Under the condition in which the GOD-bound silica gel was stored, little decrease of the activity was observed. As shown in Fig.7, the immobilized GOD kept 95% of its original activity in water over a period of 3 months. The result suggests that both denaturation of the immobilized GOD and isolation of the GOD from the silica gel hardly occurred over the period.

4. Conclusion

In the present study, polymerization of AAm was carried out in the redox system consisting of ceric ion and mercapto groups introduced onto silica gel, and the polymerization resulted in a portion of the produced polyAAm being grafted onto the surface of the silica gel. By use of a condensing agent, GOD was immobilized on the polyAAm-grafted silica gel, which was considered to be due to formation of imide linkage between the grafted polymer and GOD. It was shown that 4.3 mg of GOD was immobilized on 1 g of the polyAAm-grafted silica gel and the GOD-immobilized silica gel had an apparent activity of 50 units/g. The pH-activity profile and the temperature-activity profile of the immobilized GOD resembled those of native GOD, while the specific activity of the immobilized GOD was lower than that of the native GOD. The immobilized GOD kept 95% of its original activity in water over a period of 3 months.

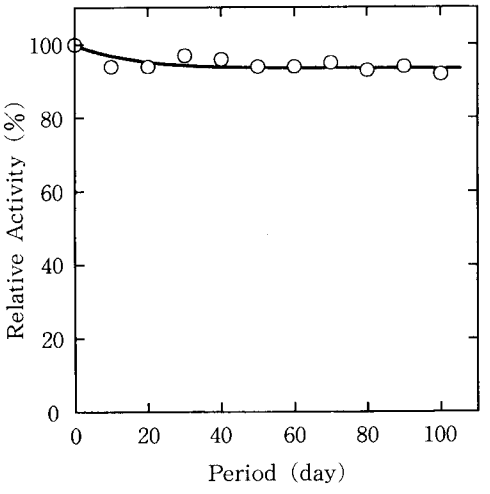

Fig. 7 Stability of GOD immobilized on polyAAm-grafted silica gel.

It was preliminarily demonstrated, as described above, that the polymer grafting technique can be applied to immobilization of enzymes on inorganic solid surfaces. However, for the practical application of the immobilized enzymes as biocatalysts, denaturation of the enzymes accompanying the immobilization must be avoided as much as possible. The problems of which sites on the enzyme molecules one can use for the immobilization without denaturation of the enzyme and what functional side-groups the grafted polymer should have for the enzyme immobilization reaction are now under investigation.

References

1) N. Fery, R, Laible and K. Hamann, Angew. Makromol. Chem., **34**(494), pp.81-109(1973).
2) R. Laible and K. Hamann, Adv. Colloid Interface Sci., **13**, pp.65-99(1980).
3) N. Tsubokawa, K. Maruyama, Y. Sone and M. Shimomura, Polym. J., **21**(6), pp.475-481(1989).
4) N. Tsubokawa, K. Maruyama, Y. Sone and M. Shimomura, Colloid Polym. Sci., **267**(6), pp.511-515(1989).
5) N. Tsubokawa, Y. Shirai, H. Tsuchida and S. Honda, J. Polym. Sci. Part A, **32**, pp.2327-2332(1994).
6) A. B. Patel, S. N. Pennington and H. D. Brown, Biochim. Biophys. Acta, **178**, pp.626-629(1969).
7) K. Mosbach, Acta Chem. Scand., **24**(6), pp.2084-2092(1970).
8) M. Valaris and W. J. Harper, J. Food Sci., **38**, pp.477-488(1973).

9) W. F. Line, A. Kwong and H. H. Weetall, Biochim. Biophys. Acta, **242**, pp.194-202(1971).

10) G. Baum, F. B. Ward and H. H. Weetall, Biochim. Biophys. Acta, **268**, pp.411-414(1972).

11) F. Borrego, M. Tari, A. Manjon and J. L. Iborra, Appl. Biochem. Biotechnol., **22**, pp.129-140(1989).

12) N. F. Almeida, E. J. Beckman and M. M. Ataai, Biotechnol. Bioeng. **42**(9), pp.1037-1045(1993).

13) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem., **193**, pp.265-275(1951).

14) P. Trinder, Ann. Clin. Biochem., **6**, pp.24-27(1969).

15) J. March, Advanced Organic Chemistry : Reactions, Mechanisms, and Structure, 4th Ed., p.419, John Wiley & Sons (1992).