Separate or Simultaneous Immobilization of Glucose Oxidase and Peroxidase on Latex Particles and Their Function

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Synopsis

Glucose oxidase and peroxidase were immobilized individually or simultaneously on aminated latex particles by using sodium *meta*-periodide and borohydride. The amount of immobilized enzymes and their activity depended on the surface potential of particles and the surface density of their own, respectively. In the simultaneous immobilization of two enzymes, the predominant immobilization of peroxidase is attributed to higher carbohydrate content in peroxidase compared with that in glucose oxidase. Simultaneously immobilized enzymes worked better in the determination of glucose than the mixture of separately immobilized ones because of the close proximity of the two enzymes.

INTRODUCTION

Enzymes are often used in immobilized form for several advantageous reasons. Enzymes immobilized on polymeric carriers can be recovered easily from the reaction systems and used repeatedly. The durability of enzymes is sometimes improved through immobilization and, as a result, such enzymes become usable under unusual conditions and can be stored for extended periods. Enzyme carriers used currently are gels, capsules, and fibers, among others. Latex particles are candidates for enzyme carriers because of their large surface area and high mobility. Several studies have been done on this subject to clarify the dependence of efficiency and activity of immobilized enzymes on immobilizing method and conditions, surface property of particles, etc.[1-5] In this study glucose oxidase (GO) and peroxidase (PO) were immobilized separately or simultaneously on several kinds of latex particles, and immobilization behavior and characteristics of immobilized enzymes were investigated. The combination of GO and PO has been used for the determination of glucose. Therefore, the availability of enzyme-bearing latex as an enzyme sensor is discussed in the latter part of this paper.

EXPERIMENTAL

Materials

Styrene (ST) was distilled at 46°C/21.5 mmHg. Acrylamide (AAm) was recrystallized from ethanol. Potassium persulfate (KPS) was used as an initiator and recrystallized from water. Two kinds of enzymes used were peroxidase (PO, Wako Chemicals Co., 100 U/mg) and glucose oxidase (GO,

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Nagase Biochemical Industry Ltd., 13 U/mg). *N*-ethyl-5-phenylisoxazolium 3′-sulfonate (Woodward's reagent K) was purchased from Tokyo Kasei Co. Sodium *meta*-periodide ($NaIO_4$) and sodium borohydride ($NaBH_4$) were obtained from Wako Chemicals Co. and used without further purification. 2,2′-Azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS(H), Wako Chemicals Co.) was used as a redox indicator.

Latex Preparation

Latex particles were prepared by soap-free emulsion copolymerization of 30 g St with 2 g AAm in 180 g water initiated by 0.432 g KPS. Whole monomers were charged in a 300 mL round-bottom flask simultaneously and the polymerization was carried out at 70°C under nitrogen for 24 h. The particles were cleaned by repeated ultracentrifugation, decantation, and redispersion in distilled water. The average diameter of particles measured from the electron micrograph was 380 nm. To investigate the particle-size effect on the activity of immobilized enzymes, larger latex particles were prepared by altering the above-mentioned reaction conditions. The details are presented in Results and Discussion.

Functional groups such as carboxyl and amino groups for binding with enzymes were introduced on the particle surface of St-AAm latices by chemical modifications. First, the amide units on the St-AAm particles were hydrolyzed to form carboxyl groups by treating 5 g particles with 5 mL 20% NaOH at 30°C for different intervals. Conductometric titration of hydrolyzed latices revealed that the amounts of on-surface carboxyl groups increased from 0.9 units/nm^2 (unhydrolyzed latex, in which a little hydrolysis had occurred during polymerization) to 3.2 units/nm^2 (15 h-hydrolyzed latex) gradually. In this connection, the initial surface density of amide units, which was estimated by the method given in our previous paper,[6] and that of sulfate groups, which were initiator fragments on polymer chain ends, were about 14 and less than 0.1 units/nm^2, respectively. The latices having the surface density from 0.9 to 3.2 carboxyl groups/nm^2 were used for immobilization of enzymes via peptide bonding.

Latex particles having amine groups were prepared by the Hofmann reaction[6] as another type of enzyme carrier. Reaction conditions and characteristics of products are described in Results and Discussion.

Immobilization of Enzyme onto Particles

Enzymes were immobilized onto particles by chemical bonding with two methods shown in Figure 1.

Immobilization via peptide bonding was performed by Wagner's method.[7] Partially hydrolyzed St-AAm particles were mixed with Woodward's reagent K at pH 8.5 and 5°C for 3 h. The amount of added Woodward's reagent K was fixed to be 1.5 times of the amount of on-surface carboxyl groups in molar basis. Then the particles were separated from the dispersion medium and added to enzyme solution at pH 7 and 5°C, kept for 3 h.

Enzymes were also conjugated onto particles by the periodate method.[8] First, enzymes were treated with 0.1 *M* $NaIO_4$ at pH 4.4 and room temperature for 20 min and dialyzed at 4°C for overnight. Then the enzymes were

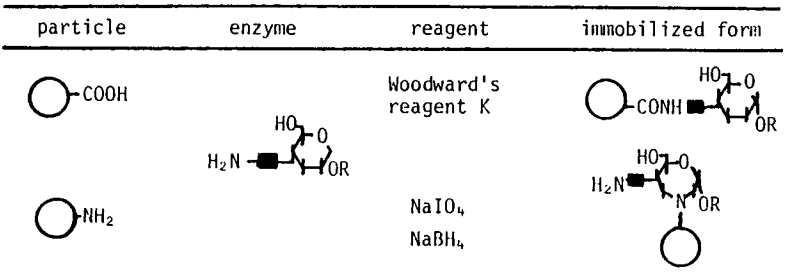

Fig. 1. Methods employed to immobilize enzymes on latex particles.

mixed with amine groups having particles which were obtained by the Hofmann reaction of St-AAm particles. The pH and temperature were kept at 9.5 and 25°C, respectively, for 2 h. The mixture was then added to 0.1 *M* $NaBH_4$ and was kept at 4°C for 2 h before cleaning.

Characterization

Determination of ionic groups on particle surface was carried out by conductometric titration.[9] The amount of enzyme bound to particles was calculated from the difference between the charged amount and the amount of enzyme remaining in the medium. The latter was measured by the Lowry method[10] in the case of a separately immobilizing system and by gel permeation chromatography (GPC) in the case of a simultaneously immobilizing system. GPC measurement was carried out using a high performance liquid chromatogram 572 (Gas Chrom. Industry Co.) with a Shodex Protein WS-800 column at flow rate 1.0 mL/min.

The activity of enzymes was measured by spectrometry based on the following reactions

$$s(\text{GO} + \text{glucose} + O_2 + H_2O \rightarrow \text{gluconic acid} + H_2O_2 \quad (1)$$

$$\text{PO} + H_2O_2 \rightleftarrows \text{Complex I} \quad (2)$$

$$\text{Complex I} + \text{ABTS(H)} \rightarrow \text{Complex II} + \text{ABTS} \quad (3)$$

$$\text{Complex II} + \text{ABTS(H)} \rightarrow \text{PO} + \text{ABTS} + 2\,H_2O \quad (4)$$

where ABTS has characteristic absorption at 420 nm. For the activity measurement of separately immobilized GO and simultaneously immobilized enzymes, 0.1 mL of enzyme-bearing latex was mixed with 9.6 mL of 1.5% glucose solution, 0.1 mL of 1 m*M* H_2O_2 and 0.2 mL of 3 mM ABTS(II). The mixture was stirred at 25°C for 20 min and its absorption was measured at 420 nm. For the activity measurement of separately immobilized PO, no glucose solution was mixed but other procedures were the same as those mentioned above.

RESULTS AND DISCUSSION

Immobilizing Method

Enzymes adsorbed physically on latex particles are more or less desorbed.[11] Therefore, a few methods to bind enzymes on particles chemically were tried. Formation of peptide bonding between enzymes and carboxylated particles using Woodward's reagent or carbodiimide was found to seriously spoil the activity of immobilized GO and PO. Their activities were less than 10% those of free enzymes. Reaction between carboxyl groups of enzymes and aminated particles led to a similar result. Therefore, ionic groups of enzymes seem to play an important role in this activity, although it is not known whether that role is direct or indirect. Alternatively, carbohydrate units of enzymes were chosen as binding sites and reacted with amine groups on particles.[8] In these cases, the activity of immobilized GO and PO was kept at about 40% that of free enzymes. The value is a satisfactory one and this binding method was adopted throughout this study.

Amount of Separately Immobilized Enzymes

The extent of Hofmann reaction of the amide groups on particle surface to form amine groups depended on the ratio of NaOCl/amide and reaction temperature if the ratio of NaOCl/NaOH and reaction time were fixed to be 1/20 and 5 h, respectively. Table I shows the amount of amine groups formed by the reaction as a function of reaction condition. The table also shows the

TABLE I
Amount of NH_2 and COOH Groups Formed by the Hofmann Reaction Under Different Conditions

$$\frac{NH_2\ (unit/nm^2)}{COOH\ (unit/nm^2)}$$

Reaction temperature (°C)	[NaOCl]/[$CONH_2$] (M/M)			
	0.1	0.4	0.7	1.0
0			0.31 / 0.25	
4		0.48 / 0.52	0.56 / 0.31	
20	0.29 / 0.51	0.40 / 0.34	0.83 / 0.52	1.09 / 0.60
35		0.49 / 0.54	0.76 / 0.46	1.27 / 0.54
50		0.28 / 0.50		
70			0.61 / 0.71	

[NaOCl]/[NaOH] (M/M) = 20, reaction time 5 h.

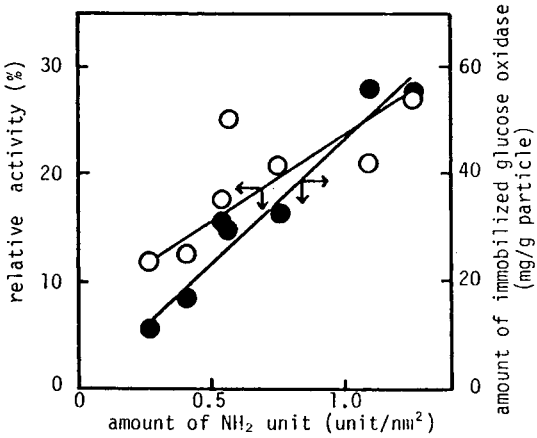

Fig. 2. Effect of NH_2 density on the amount and the relative activity of immobilized glucose oxidase. (●) amount of immobilized GO; (○) percentage of activity of immobilized GO to that of the same amount of free GO; charged GO: 154 mg/g particle.

amount of carboxyl groups formed by hydrolysis competing with the Hofmann rearrangement.

It was found that the amount of immobilized enzymes depended on the surface density of amine groups on particles but not on that of carboxyl groups. The dependence of immobilized amount on the amine density is shown in Figures 2 and 3. The amount of immobilized enzymes was supposed to increase with increasing density of the binding sites on particles, NH_2 groups. This was the case for GO immobilization but not for PO immobilization. The unusual dependence for PO seems to be explained in terms of the electrostatic interaction between an enzyme molecule and a particle. Namely, the fact that the electrophoretic mobility of PO-bearing particles became mostly constant regardless of that of particles before combining enzymes, as

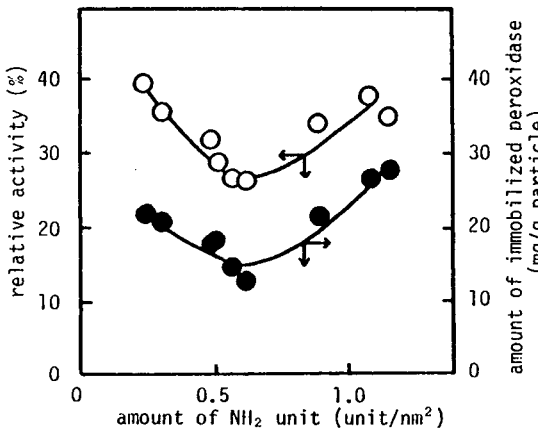

Fig. 3. Effect of NH_2 density on the amount and the relative activity of immobilized peroxidase. (●) amount of immobilized PO; (○) percentage of activity of immobilized PO to that of the same amount of free PO; charged PO: 44 mg/g particle.

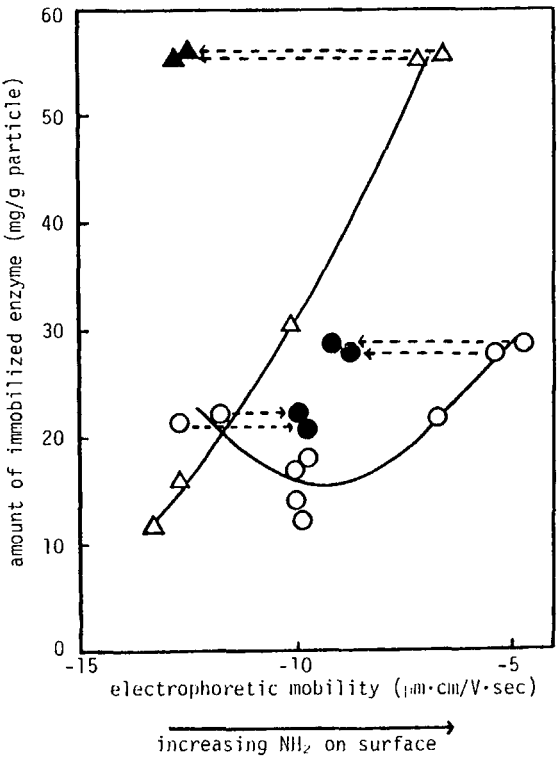

Fig. 4. Dependence of the amount of immobilized enzymes on the electrophoretic mobility of latex particles at pH 9.5 (△▲) GO; (○●) PO; (△○) electrophoretic mobility (EPM) of particle having no enzymes; (▲←, →●, ●←) EPM shift caused by enzyme immobilization; charged amount: GO 154 mg, PO 44 mg/g particle.

shown by closed symbols in Figure 4, would indicate that the difference in surface potential between enzyme and particle can be a factor in determining the extent of immobilization. The result obtained in GO immobilizing system, shown in Figures 2 and 4, supports the above speculation.

Activity of Separately Immobilized Enzymes

Relative activity in Figures 2 and 3 measures the percentage of activity of immobilized enzymes against that of the same amount of free enzymes. According to the results shown in the figures, the activity of immobilized enzymes increases with increasing amount of immobilized enzymes. A different dependence of activity on immobilized amount was observed in Figure 5 which indicates that too great an amount of immobilization reduces the specific activity of the enzyme. The calculated molecular area of immobilized GO ranged from 48 to 384 nm^2/molecule whereas that of native GO is supposed to be about 120 nm^2/molecule. The maximum activity was observed when GO was immobilized to occupy approximately the same surface area with the native one. It was concluded that too loose or too tight immobilization causes a decrease in the specific activity of enzyme, perhaps due to expanded or compressed conformation. In the case of PO, the molecular area of immobilized

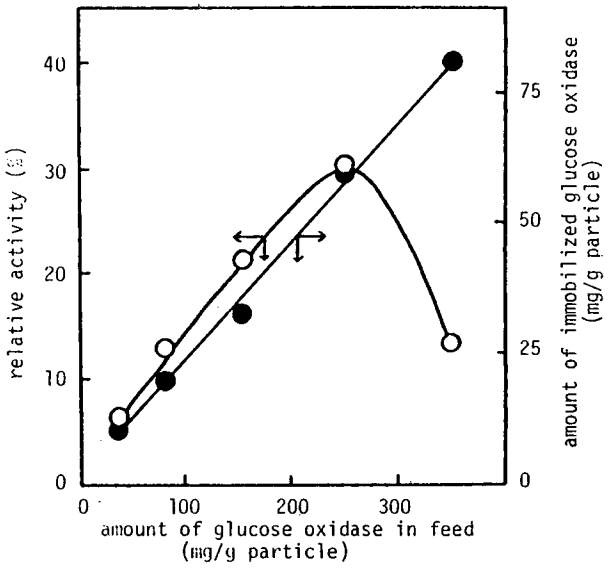

Fig. 5. Effect of charged amount of glucose oxidase on the amount and the relative activity of immobilized enzyme. (●) amount of immobilized GO; (○) percentage of activity of immobilized GO to that of the same amount of free GO; charged GO: 154 mg/g particle.

enzyme ranged from 40 to 85 nm^2/molecule and that of native one was 50 nm^2/molecule. PO was not immobilized too tightly and, therefore, the specific activity of immobilized PO increased monotonously with an increase in its surface density as shown in Figure 3.

Amount of Simultaneously Immobilized Enzymes

A certain amount of GO and PO, 154 mg and 22 mg, respectively, were added together to 1 g of several latices having different NH_2 densities. The amount of immobilized GO and PO is shown in Figure 6. The dependence of the amount of immobilized GO on the NH_2 content was similar to that in the separately immobilized GO. On the other hand, the shape of immobilized amount versus NH_2 density curves showed no resemblance between separate and simultaneous immobilizations of PO. It is probable that, in the simultaneous immobilization, the competitive binding of GO would change the surface potential of particles and, consequently, affect the electrostatic interaction between particle and PO molecule.

The result in Figure 6 indicates that about 80% of PO charged was immobilized regardless of the surface structure of particles but the efficiency of GO immobilization was not very high, especially in the case of immobilization on particles of low NH_2 density. The difference in immobilization efficiency between the two enzymes was also observed in Figure 7, where different amounts of PO and a certain amount of GO were added to a latex. Low immobilization efficiency of GO and high efficiency of PO seems to be explainable from the difference in content of carbohydrate moiety between the two enzymes. Carbohydrate fractions in GO and PO molecules are 0.11 and 0.18, respectively.[12] Specifically, PO has more binding sites and shows higher immobilization efficiency.

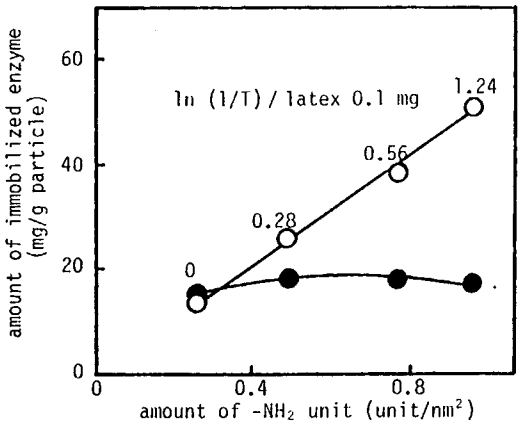

Fig. 6. Effect of NH_2 density on the amounts of simultaneously immobilized GO and PO and the sensitivity for glucose determination. (○) amount of immobilized GO; (●) amount of immobilized PO; numerical value: ln(1/T)/0.1 g enzyme-bearing latex; (T) absorbance change due to ABTS formation accompanying with enzymatic decomposition of glucose [eqs. (1–4)]; charged GO: 154 mg; charged PO: 22 mg/g particle.

Activity of Simultaneously Immobilized Enzymes

The activity of simultaneously immobilized GO and PO was evaluated by glucose sensitivity determination using ABTS as a color-producing reagent through the redox reaction. The increment in absorbance at 420 nm in the course of reaction was regarded as a measure of sensitivity. The numerical values for each system are presented above each plot in Figure 6 and above each bar in Figure 7.

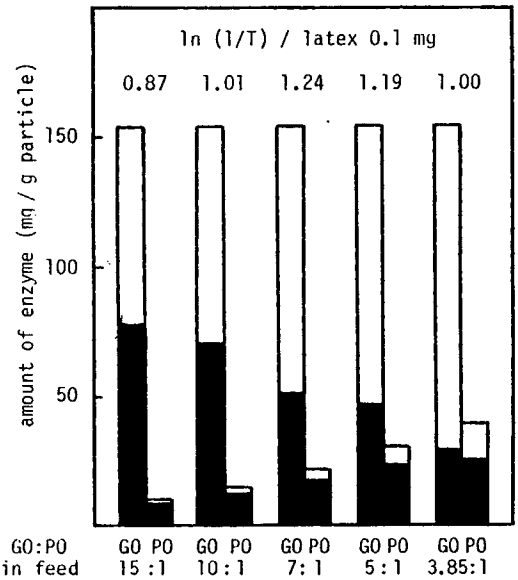

Fig. 7. Effect of ratio of charged GO and PO on the amounts of immobilized enzymes and the sensitivity for glucose determination. (▯) amount of charged enzyme; (■) amount of immobilized enzyme; numerical values: same with those in Figure 6.

According to the data shown in Figure 6, sensitivity increased with increasing amount of immobilized GO when a fixed amount of latex particles were used. The highest sensitivity was recorded using particles carrying GO and PO at about 3 : 1 on weight basis or 0.39 : 1 in terms of enzymatic activity in the range examined. It is probable that a higher sensitivity will be attained if more GO is immobilized on the particles judging from the ratio in enzymatic activity although no such trials have been done. Murachi et al.[13] reported that sensitivity of GO–PO system in gel column increased with GO/PO weight ratio up to 4 : 1 or with the enzyme-activity ratio up to 3 : 1 where they used much more active GO (70 units/mg) than ours. Data in Figure 7 were obtained using several particles on which the immobilized amounts of both enzymes were different from each other. In this series, the highest sensitivity was recorded at the weight ratio 3 : 1 of GO and PO.

Advantages of simultaneous immobilization were determined by comparing the sensitivity of glucose determination between a latex bearing GO and PO simultaneously and a mixture of GO-bearing particles and PO-bearing ones. The result is shown in Figure 8. Both enzyme-bearing particles recorded a higher sensitivity than the mixture of enzyme-bearing particles although the amount of enzyme used in the former was half of the latter's.

Efficient cooperation between simultaneously immobilized enzymes is attributed to close location of two enzymes which promotes the transfer of H_2O_2 from GO site to PO site. The average distance between the centers of two

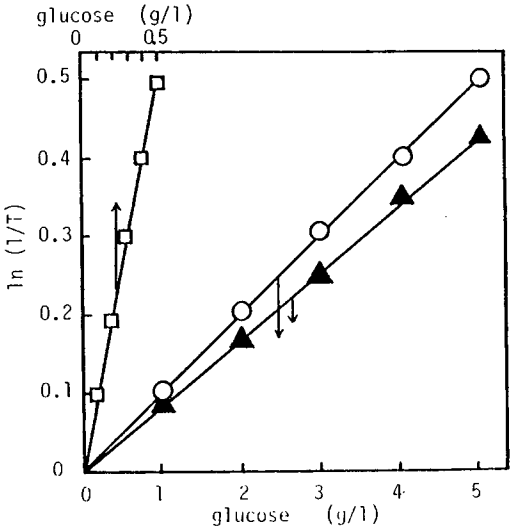

Fig. 8. Sensitivity of enzyme(s)-bearing latices for glucose determination

Immobilization mode	Amount used for determination		
	GO (μg)	PO (μg)	Particle (mg)
▲ separate	3.0	0	0.051
	0	1.0	0.036
○ simultaneous	1.5	0.5	0.030
□ simultaneous	10.0	3.3	0.20

adjacent molecules of simultaneously immobilized enzymes on the particle surface was roughly calculated to be about 7 nm. It should be mentioned that this value is rather too small for the separately immobilized enzymes to exhibit their maximum activity.

Higher sensitivity or higher absorbance can be detected by using more latex particles as shown in the upper left corner of Figure 8. The concentration level to be measurable under such conditions corresponds to that of an enzyme electrode.[14] Even in such a case, the amount of latex particles used was so small that the absorption measurement was not disturbed by latex particles and could be done in the presence of latex particles. The particles could be recovered from a reaction system by centrifugation and reused. It is concluded from these results that the simultaneously immobilized system can be a kind of glucose sensor due to its simplicity, high sensitivity, and reusability.

Immobilization of Enzymes on Larger Particles and Their Activity

In the experiments done hitherto, latex particles of 380 nm diameter were employed. But, it is obvious that the larger the carrier, the easier the recovery becomes. Therefore, larger particles were prepared and applied as the other carriers for enzymes. Large particles were obtained by modifying polymerization conditions as follows; dispersion medium was converted from water to 10% aqueous methanol and AAm was prepolymerized for 1 h before St was charged. The resulting particles had 1010 nm diameter but only small amounts of amide units, which were to be converted into amine binding sites on their surface. An additional charge of 2 g AAm at 70% conversion enabled the formation of monodisperse particles having 980 nm diameter and a sufficient number of amide units.

Simultaneous immobilization of GO and PO on these large particles could be done in the same manner as that for small particles. But the sensitivity of glucose determination was found to be less than one-fourth that of the corresponding small particle system, although the surface density of enzymes was almost the same. Moreover, large particles brought about nonnegligible turbidity. It was concluded that large particles are unsuitable carriers for this type of glucose sensor. Some means other than enlargement of particles must be sought for easy recovery of enzyme carriers from the reaction system, for example, use of high density carrier or recovery by electric or magnetic forces using highly charged or magnetic particles, would be worth trying.

CONCLUSIONS

Glucose oxidase and peroxidase were immobilized onto aminated latex particles using their carbohydrate units as binding sites. The amount and activity of immobilized enzymes depended on the surface potential of particles and the surface density of immobilized enzymes, respectively. Simultaneously immobilized enzymes on single particles indicated a higher sensitivity for glucose determination than the mixture of separately immobilized ones.

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