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Immobilization of Glucose Oxidase and Peroxidase and Their Application in Flow-Injection Analysis for Glucose in Serum

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ABSTRACT

Glucose oxidase (GOD) and Horseradish peroxidase (HRP) were covalently coupled to alkylamine controlled pore glass by means of glutaraldehyde. About 700–800 U/g of immobilized GOD and 300–400 U/g of immobilized HRP were obtained. Some factors of affecting enzyme immobilization were discussed.

The immobilized enzymes were packed into a plastic tube and used in flow-injection analysis (FIA) for glucose in serum. A good linearity range was observed for this immobilized enzyme system at 20 mg/mL to 1000 mg/dL D-glucose, the recovery was 95.4–103.5%, the within-batch imprecision was 0.8–2.2%, and the between-batch imprecision was 2.2–4.2%. More than 100 samples were measured within an hour. One enzyme column with five units of immobilized GOD and HRP, applied for 50 assays/d, has been used for more than 2 mo.

Index Entries: Glucose oxidase; Horseradish peroxidase; immobilization on controlled pore glass; flow-injection analysis for glucose in serum.

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INTRODUCTION

Immobilized enzymes have attracted much attention in the past decade and their applications developed rapidly in analytical and clinical chemistry (*1,2*).

Glucose oxidase (GOD) and horseradish peroxidase (HRP) were successfully immobilized on controlled pore glass (CPG) by cross-linking with glutaraldehyde, and the immobilized enzyme reactor was applied in a flow-injection analysis (FIA) system for blood glucose determination. The GOD-catalyzed conversion of β-D-glucose to gluconic acid and hydrogen peroxide is broken down to water and oxygen by HRP in the presence of an oxygen receptor. The procedure is most commonly used in clinical laboratories to measure blood glucose.

The method described here is simple, rapid, and particularly effective. The sample containing glucose is injected into and mixed with the carrier solution that is allowed to flow through the enzyme reactors. In the course of the enzymatic reaction, the oxygen receptor is oxidized to a colored product. The change in absorbance at 505 nm is measured, and the concentration of glucose is calculated.

MATERIALS AND METHODS

Instruments

1. FIA-1A/BSOIF Flow-Injection Analysis System (Beijing, China) was used for glucose determination.
2. Hitachi 150-20 Spectrophotometer (Japan) was used for determination of enzyme activity.
3. M10 Biochemical Analyser (Instrument Laboratory, USA) was used for determination of blood glucose by the GOD method in comparison studies.

Reagents

GOD: crude solution, obtained from the Institute of Microbiology, Academia Sinica, was concentrated to 15 mg protein/mL or above (80–140 U/mg protein) and stored at 4°C.

HRP: Horseradish peroxidase (Institute of Biochemistry, Academia Sinica), $Rz = 3.0$, 2000–3000 U/mg, 8501014.

CPG: SB46-46, (mean pore diameter 750Å) was obtained from the Institute of Silicate Chemistry, Academia Sinica, Shanghai, China, and PG-200 (120–200 mesh, mean pore diameter 170–2000Å) were obtained from Sigma, St. Louis, MO.

Buffer solution: Phosphate buffer, 0.1 mol/L, pH 6.5 and 7.5; carbonate buffer, 0.2 mol/L, pH 10.0; and acetate buffer, 0.1 mol/L, pH 5.0.

Carrier solution: 0.05 mol/L phosphate buffer, pH 6.0, which contains phenol (0.5 g/L), 4-aminoantipyrine (50 mg/L), and sodium azide (0.5 g/L), was used as carrier solution in flow-injection analysis for glucose determination.

Glucose standards: β-D-Glucose (AR) was dried in an oven at 80°C for 3 h and 2% solution of glucose in 0.14% (w/v) benzoic acid solution was made as stock solution. A series of standard working solutions with glucose concentrations in the range of 0–1000 mg/dL, were prepared by diluting the stock solution with 0.14% benzoic acid solution and stored at 4°C.

GOD Kit: used for glucose determination (Institute of Microbiology, Academy of Science, Hebei, China).

Other reagents: Glutaraldehyde, 25% (w/v) electron microscopy grade; O-Dianisidin, Fluca AG, Chem. Fabrik CH-9470 Buchs; and γ-Aminopropyltriethoxysilane (CP, Gaixian Chemical Plant, Liao Ning, China); other chemicals were of analytical grade.

Methods

Silanization of CPG: 1 g of CPG was immersed in 5 mL of 2% solution of γ-aminopropyltriethoxysilane in acetone for 10 min at room temperature. Excess liquid was decanted off and the glass was heated at 80°C for 5–6 h. The resulting product is an alkylamine glass.

Immobilization of GOD: To 0.2 g of alkylamine glass, 2 mL of 0.1% (w/v) solution of glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.5, was added. The mixture was placed under vacuum in a dessicator for 15 min and then stood for 45 min at room temperature. The glass was thoroughly washed with distilled water. To the glass, 0.4 mL of GOD solution (15 mg/mL) and 0.1 mol/L phosphate buffer, pH 6.5, was added. The reaction took place in a vacuum dessicator for 10 min and then at 4°C overnight. The immobilized enzyme was washed with 800 mL of distilled water and stored at 4°C in distilled water.

Immobilization of HRP: Two milliliters of 2.5% solution of glutaraldehyde in 0.1 mol/L, pH 7.5 phosphate buffer was added to 0.2 g of alkylamine glass. The mixture was placed under vacuum in a dessicator for 15 min and then stood for 45 min at room temperature. The glass were washed with distilled water. To the glass, 0.2 mL of HRP solution (10 mg/mL) and 0.05 mL of 0.2 mol/L, pH 10.0 carbonate buffer were added. The reaction was performed at 4°C overnight that included 10 min in a vacuum dessicator. To the reaction mixture, 1.8 mL of distilled water and 0.1 mL of 4.0 g/L sodium borohydride were added. The reaction was continued at 4°C for another 2 h and then washed with distilled water until no enzyme activity was detected in the drained water. The immobilized enzyme was stored at 4°C in distilled water.

Preparation of the Immobilized Enzyme Reactor: The immobilized GOD and HRP were packed into a plastic tube (2.5 mm id, length 25 mm)

and both ends were sealed with a nylon net. The packed-bed reactor contains 5–10 U GOD and HRP.

Determination of Enzyme Activity: The spectrophotometric method using O-dianisidine as a chromogenic oxygen acceptor was used to determine the activity of GOD. For native enzyme, the assay procedure was performed according to ref. (3). For immobilized enzyme, the following procedure was used: to 30–50 mg of immobilized GOD, 52 mL of 0.1 mol/L, pH 5.0 acetate buffer, 0.43 mL of 10 g/L solution of O-dianisidine in methanol and 2.0 mL of POD solution (60 U/mL) were added. To the mixture, 6.0 mL of 1 mol/L glucose was added. After 1 min, 3 mL of the reactants were taken out and mixed with 0.2 mL of 0.5 mol/L sulfuric acid immediately. The absorbance of the reaction solution at 460 nm was read in a spectrophotometer and the activity of immobilized GOD was calculated as following

$$(U/g) = \frac{\Delta A460\ nm/min}{11.3} \times \frac{Reaction\ volume\ (mL)}{Enzyme\ added\ (mg)} \times 1000$$

One unit (U) of GOD activity is defined as the amount of enzyme that liberates 1 μmol hydrogen peroxide/min under the conditions described above.

HRP Activity Determination: The activity of soluble enzyme was assayed by the method described in ref. (4). One unit of HRP activity is defined as the amount of enzyme that consumes 1 μmol of peroxide/min at 25°C. The activity of immobilized enzyme was assayed by the following procedure: 60 mL of 0.05 mol/L, pH 6.0 phosphate buffer and 0.5 mL of 10 g/L solution of O-dianisidine in methanol were added to 30–50 mg of immobilized HRP. To the mixture, 0.6 mL of 0.3% hydrogen peroxide was added. After 1 min, 3.0 mL of reactants were taken out to which 0.1 mL of 1 mol/L HCl was added immediately. The change of absorbance at 460 nm was measured and the activity of immobilized HRP was calculated as follows:

$$(U/g) = \frac{\Delta A460\ nm/min}{11.3} \times \frac{Reaction\ volume\ (mL)}{Enzyme\ added\ (mg)} \times 1000$$

Protein Determination: The protein of GOD was determined according to the method described by Lowry et al. (5) and that of HRP was determined by the spectrophotometric method (6).

RESULTS AND DISCUSSIONS

Immobilization of Enzymes

Silanization of CPG: The procedure of silanization of CPG varies with the solvents used for dissolving silane reagent: (1) distilled water: the reaction pH must be adjusted to 3.5 precisely in the silanization proce-

Table 1
Effect of Glutaraldehyde Concentration
on The Efficiency of GOD Immobilization

Glutaraldehyde concentration, %	Porous glass, g	Enzyme added, mg	Enzyme coupled, mg	GOD-CPG activity, U	Relative activity, %
2.0	0.2	3.52[a]	3.48	25	8.7
1.0	0.2	3.52	3.45	31	10.7
0.5	0.2	3.52	3.48	38	13.0
0.25	0.2	3.52	3.45	54.5	19.0
0.13	0.2	3.52	3.46	65	22.6
0.10	0.2	3.54[b]	3.46	49	21.1
0.05	0.2	3.54	3.44	47	20.2
0.02	0.2	3.54	3.35	24	10.7
0.01	0.2	3.54	3.33	23	10.2

[a]GOD: 83 U/mg.
[b]GOD: 67.5 U/mg.

dure (*7*); (2) Toluene: the reaction was carried out under reflux at 110°C in a liquid paraffin bath (*8*). The silanized products are stable but the procedure is tedious; (3) Acetone: the procedure is comparatively simple and the result is good enough (*9*). We used the acetone as solvent and modified the reaction time to 5–6 h at 80°C, which is sufficient to achieve maximum silane-surface stability (*10*).

Preparation of Activated Intermediates: An activated aldehyde intermediate is readily prepared by reacting glutaraldlehyde with an alkylamine glass. In our study, the reaction was carried out in 0.1 mol/L phosphate buffer solution, pH 7.5.

It is very interesting that the concentration of glutaraldehyde can strongly affect the activity of immobilized GOD. In the previous study, 2.5% (w/v) glutaraldehyde was commonly used. However, the present study showed that high concentrations of glutaraldehyde may decrease the apparent activity of immobilized GOD and the better result was obtained by using low concentrations of glutaraldehyde (Table 1). It is supposed that the more linkages formed between one enzyme molecule and the support, the lower apparent activity expressed in the immobilized enzyme. This phenomenon may also be related to the size of enzyme molecules and the nature of support. In our experiment, 0.1% glutaraldehyde was used for GOD immobilization and 2.5% for HRP.

Enzyme Coupling Reaction: In a typical coupling procedure, the enzyme is immobilized on the support in neutral solutions and a fairly stable linkage can be formed. In the present experiment, the immobilization of GOD was carried out in pH 6.5 phosphate buffer and that of HRP in pH 10.0 carbonate buffer. The imine linkage between enzyme and support formed in alkaline solution is somewhat unstable, but the stability of immobilized HRP can be improved by reducing the imine using sodium borohydride (*11*).

The activity of immobilized enzyme was affected by the quantity of soluble enzyme added. Within a certain range, the activity of immobilized enzyme can be improved via enhancing the quantity of enzyme added, but the relative activity (the ratio of activity expressed to activity coupled) was decreased at the same time. To 0.2 g of CPG, 5–6 mg of GOD (0.4 mL) or 1–3 mg of HRP (0.2 mL) was added in our study.

The pore size and the particle size of the glass affect the coupling of the enzyme to CPG. The result shows that the superior activity of immobilized enzyme is obtained with pore diameter 240 Å for GOD and 170 Å for HRP. In the following study, GOD was coupled to the glass SB46-46 (100–200 mesh) or PG-240-200 and HRP was coupled to the glass PG-170-200.

In the present procedure, 700–800 U/g of the immobilized GOD and 300–400 U/g of immobilized HRP can be obtained. The relative activities of immobilized GOD and HRP were 15–25 and 20–30%, respectively. Stored at 4°C in distilled water, no loss of enzyme activity was observed within 3 mo.

APPLICATION OF IMMOBILIZED ENZYMES

In this study, the immobilized enzyme reactor was combined with a flow-injection analysis (FIA) system and used for the blood glucose determination. The layout of the system is shown in Fig. 1; the carrier solution was pumped into the teflon tubing (0.5 mm id) at a flow rate 3–3.5 mL/min. The length of mixture coil was 0.7 m and the reaction temperature was kept constant in a water bath at 30°C. The absorbance change of the solution in the flow cell (70 μL, 10 mm path-length) was monitored at 505 nm. Glucose standards and serum samples (4 or 6 μL) were introduced into the system by means of a rotation sampling valve. The results of this experiment are as follows:

1. Precision: The within-batch imprecision ranged from 0.8 to 2.2% and the between-batch imprecision for 21 assays in a 5-d period ranged from 2.2–4.2% (Table 2).
2. Accuracy: The percentage of recovery of the method ranged from 95.4 to 103.5%. It is good enough for routine determination of serum glucose in clinical chemistry (Table 3). A comparison experiment was carried out in 102 serum samples from patients suffering from various diseases (including hemolyzed and jaundice samples), both FIA (enzyme reactor) and a M10 Analyzer (GOD kit) were used for glucose determination. The latter is daily used as an analyzer for serum glucose by using the glucose oxidase method in a clinical laboratory. The correlation between the results obtained from the two methods are given in Fig. 2 and can be summarized as follows: $Y = 1.051X - 6.899$ and $r = 0.998$ (Y, enzyme reactor; X, used GOD routine method).

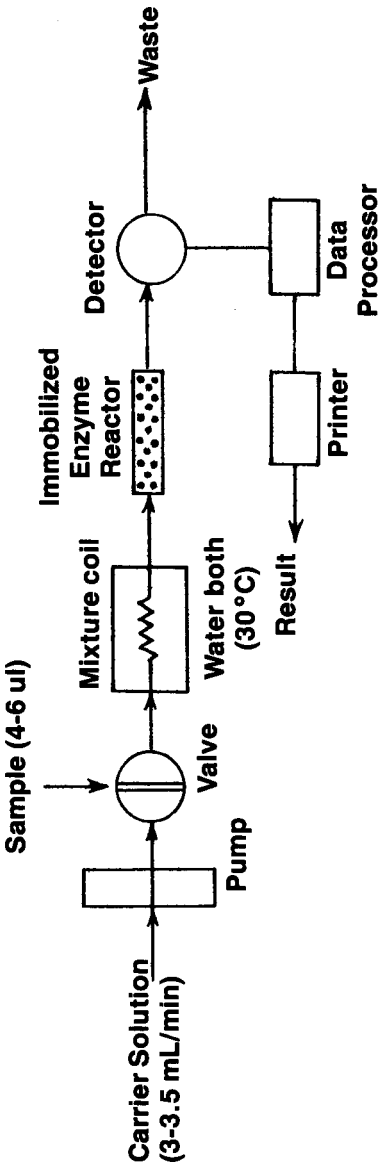

Fig. 1. Diagram of FIA for glucose determination using GOD and HRP column reactor.

Table 2
Precision Data
of Flow-Injection Analysis for Blood Glucose

Serum	X, mg/dL	Within batch[a] n	SD	CV %
1	61.63	27	1.38	2.2
2	114.28	28	1.195	1.00
3	240.93	30	1.91	0.80
		Between batch[b]		
1	73.77	21	3.09	4.19
2	136.02	21	5.44	4.00
3	268.77	21	6.03	2.24

[a]Sample: 4 μL of controlled serum.
[b]Sample: 6 μL of controlled serum, 21 assays over 5 d.

Table 3
Recovery of Glucose Added in Serum[a]

Serum	Original, mg/mL	Added, mg/mL	Measured, mg/mL	Recovery, %
1	74.31	148.15	227.6	103.5
2	83.22	76.92	161.9	102.3
3	82.98	29.56	112.2	98.9
4	49.81	29.56	80.41	103.5
5	159.3	29.56	187.5	95.4

[a]Sample: 6 μL of mixture serum.

3. Calibration Curve of Glucose: A good linear calibration curve was obtained for this immobilized enzyme system at 20–1000 mg/dL β-D-glucose. This effectively covers the normal range of blood glucose (70–110 mg/dL), as well as hypo- and hyperglycemia levels (Fig. 3). The typical response peaks of injections of glucose samples and control sera were shown in Fig. 4.

By the present method, the blood samples can be continuously analyzed for glucose at a rate of 100 samples or even more per hour. The carryover of this system was 0.3 and 1.5% at a sampling rate of 60 and 120 times/h, respectively. The sample volume injected into the system was 4 or 6 μL, with less than 50 μL being required for each determination. If 50 samples were analyzed daily, one enzyme reactor that contains 5 U of immobilized GOD and HRP can be used for over 2 mo. The enzyme reactor was stored in dw at 4°C when it was not in use (0.5 g/L sodium azide in phosphate buffer, pH 7.0, should be required for long-term storage).

It is obvious that the immobilized GOD and HRP reactor used in FIA technique for determination of glucose in serum is very simple, cheap, and suitable in a clinical laboratory for routine or emergency use. The

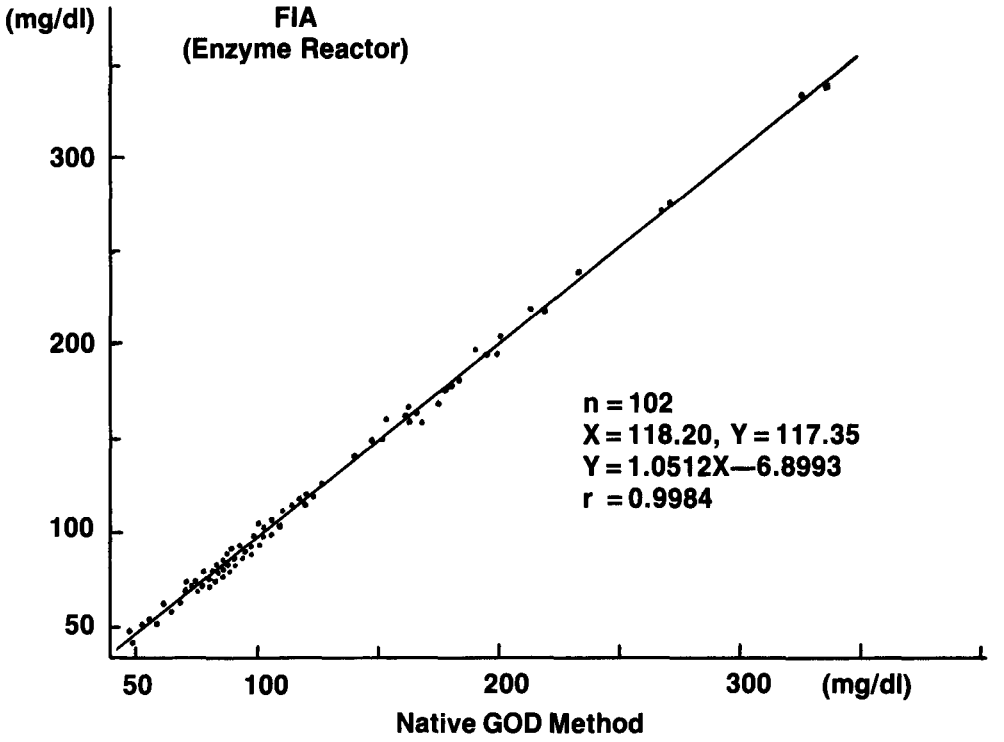

Fig. 2. Correlation and regression line for serum glucose measured by immobilized enzyme method against serum glucose measured by native GOD routine method.

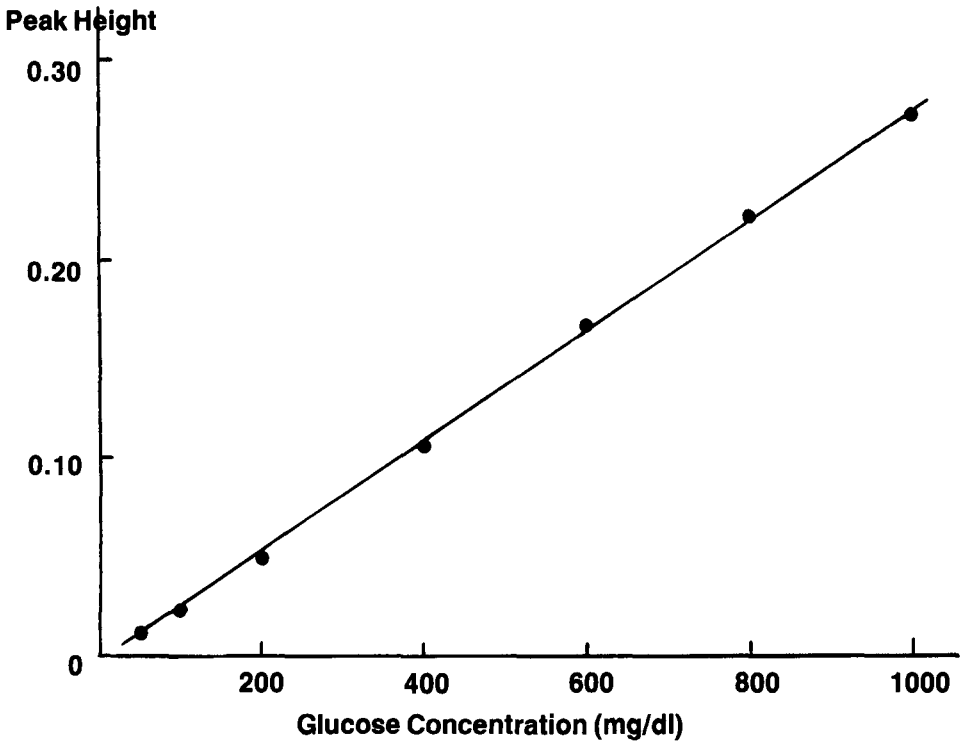

Fig. 3. Calibration curve for glucose using FIA system.

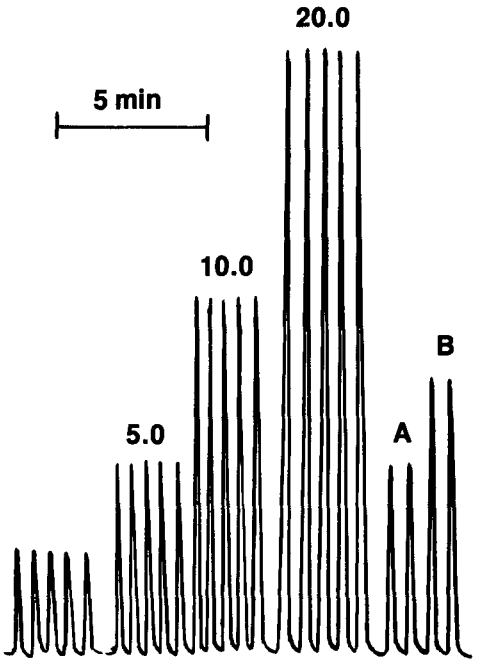

Fig. 4. Recorder tracing of FIA for glucose analysis using the immobilized enzyme (Glucose concentration: mmol/L; A, B: controlled serum).

optimization in the design of packed-bed enzyme reactors for FIA and other related research results will be discussed in a separate paper.

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