

Glucose-sensitive insulin-releasing molecular systems

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Abstract: Three types of molecular systems for insulin release in response to glucose were designed and synthesized. i) Chemical valve was synthesized by immobilization of glucose oxidase onto poly(acrylic acid)-grafted porous membrane. ii) Membrane device was constructed by immobilization of insulin through disulfide linkage with combination of glucose dehydrogenase and electron mediators. iii) Enzyme device was made by coupling glucose oxidase and insulin through disulfide linkage.

INTRODUCTION

Glucose-sensitive insulin-releasing system is required for diabetes therapy. Figure 1 shows the insulin levels throughout the day in a group of nondiabetics. There is an almost constant basal supply during the night and between meals, which maintains glucose levels by retaining and controlling hepatic glucose output. At meal-times, glucose input from the gut increases and triggers a boost of insulin from the B cells of the islets of Langerhans. The augmented insulin level affected the dose-response curves of glucose disposal in the periphery, protein synthesis, lipogenesis, etc. as well as inhibiting liver glucose output and increasing glycogen deposition.

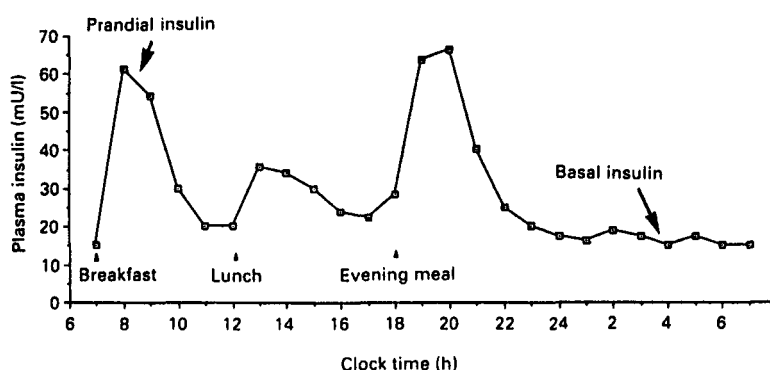


Figure 1 Plasma insulin levels throughout the day in non-diabetic subjects. Note 'basal' insulin secretion between meals and during the night, and insulin boosts at meal-time ('prandial' insulin).

For the insulin administration, the feedback (closed-loop) system, which, in response to blood glucose levels, modulates or triggers the release of insulin, have been investigated (1-3). The approaches are categorized into three, as shown in Figure 2.

Artificial Pancreas

I. Immuno-isolation of pancreas cell by encapsulation

II. Electric combination of glucose sensor and insulin injector

Glucose Sensor \longleftrightarrow Microcomputer \longleftrightarrow Insulin Injector

III. Molecular system

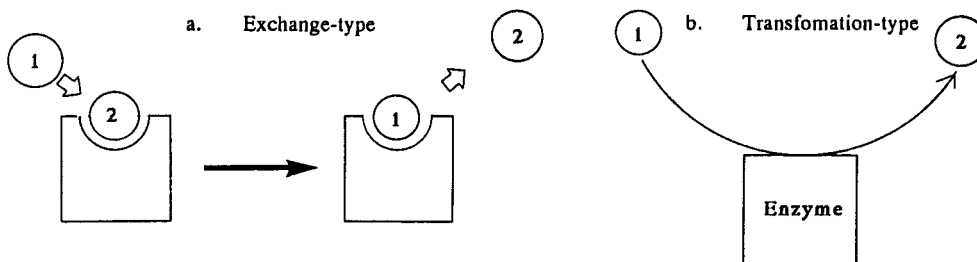


Figure 2 Classification of ideas to construct artificial pancreas.

One is encapsulation of pancreas islet cell or the derivative cells in an inert matrix whose purpose is immunoisolation. The second is an electromechanical system consisting of a glucose sensor and an insulin injection pump. The third is a molecular systems, which are further categorized into signal-exchange and signal-transformation types. In the former cases, lectin- or borate-saccharaide interaction was employed (4,5). In the latter cases, glucose-sensitive enzymes such as glucose dehydrogenase and glucose oxidase, which were usually used for glucose sensors, were utilized (6-10). Because the enzymes transformed the chemical signal of glucose to physical signals such as pH change or redox reaction, they were immobilized on pH- or redox-sensitive molecular systems. We have developed three molecular systems using the glucose sensitive enzymes.

CHEMICAL VALVE

We immobilized glucose oxidase (GOD) on a pH-sensitive chemical valve, which we devised (11-13). The valve was synthesized by grafting poly(acrylic acid) on a porous polycarbonate membrane. The graft chain extended at high pH to close the pore, and at low pH the chain shrank to open the pore. As illustrated in Figure 3, the immobilized GOD catalyzed glucose to gluconic acid, which reduced pH of solution and the pore opened.

Figure 4 shows that the insulin release rate increased after glucose addition. Previously glucose oxidase was immobilized on pH-sensitive membrane which was composed of hydrogel containing cationic groups by two groups (7,8). The gel membranes swelled at high pH to release insulin, and shrank at low pH not to release insulin. However, the response time is not so fast in such system because the insulin has to diffuse in polymer chains not pores. The characteristic of chemical valve system is rapid response.

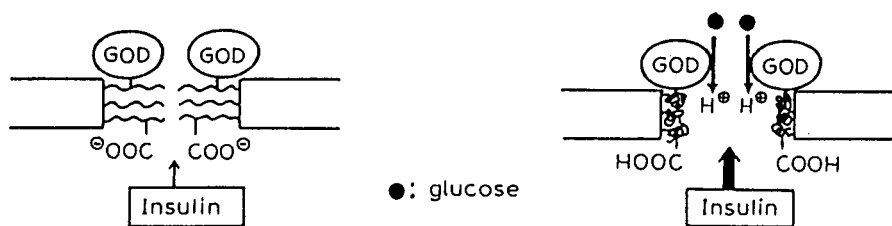


Figure 3 Principle of controlled release system on insulin: GOD, glucose oxidase. **Left:** in the absence of glucose, the chains of poly(acrylic acid) grafts are rod-like, lowering the porosity of the membrane and suppressing insulin permeation. **Right:** in the presence of glucose, gluconic acid produced by GOD protonates the poly(acrylic acid), making the graft chains coil-like and opening the pores to enhance insulin permeation.

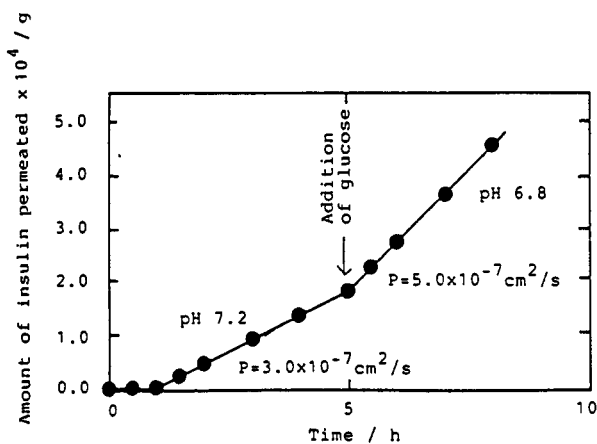


Figure 4 Permeation of insulin through poly(acrylic acid)-grafted membrane in 0.1 M Tris-HCl-buffered solution. The concentration of added glucose is 0.2 M.

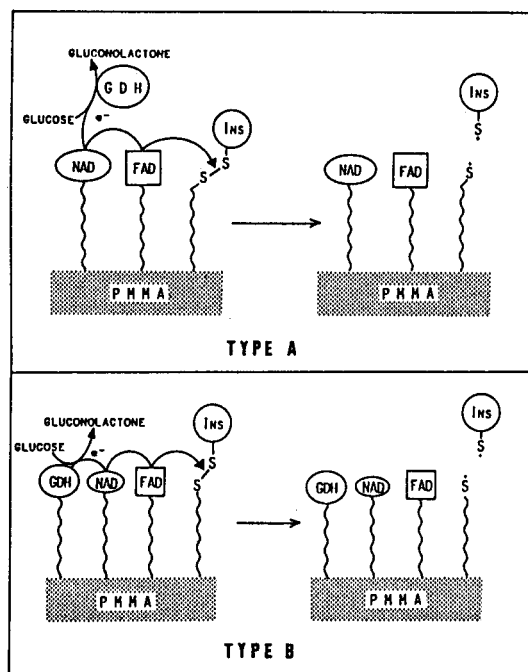


Figure 5 Design of glucose-sensitive insulin releasing membrane system based on redox reaction. Ins, insulin; S-S, disulfide bond of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB); GDH, glucose dehydrogenase.

MEMBRANE DEVICE

Above-mentioned valve systems have, however, still disadvantages in their insensitive on-off response: the slow diffusion of insulin through the membrane in spite of through pore, and the release of insulin even in the absence of glucose. Therefore, we synthesized another type of insulin releasing system using redox reaction (14,15). The principle is shown in Figure 5. As a glucose-sensitive enzyme glucose dehydrogenase (GDH) was used, because it is known that the enzyme contains the active site near the surface to transmit the generated electron to enzyme co-factors NAD and FAD. (GOD has the active site in the deep site not to transfer the electron to the surface without modification.)

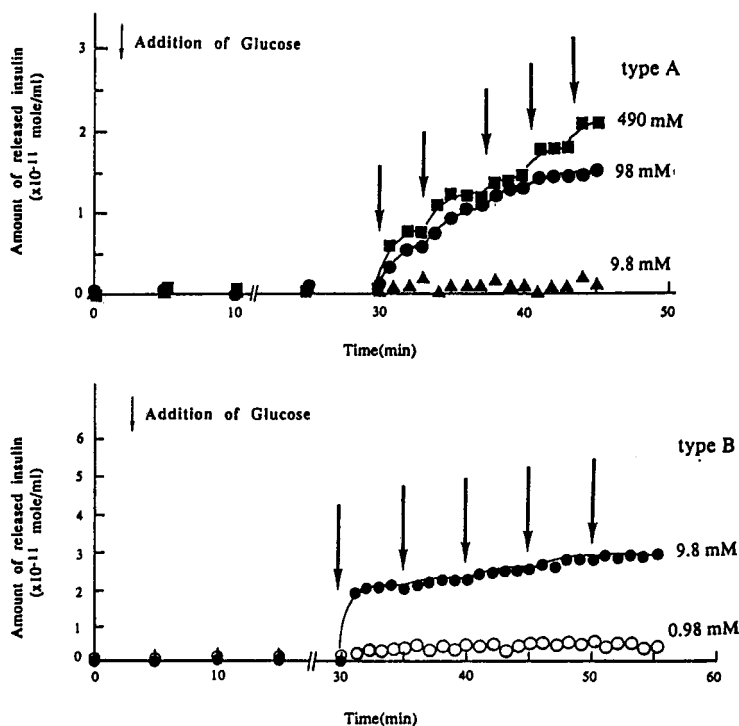


Figure 6 Release of insulin from composite membrane devices (types A and B) in response to various concentrations of glucose.

The system was in principle composed of insulin immobilized to an insoluble polymer membrane through a disulfide bond and GDH. When glucose molecules were present in solution, GDH oxidized the glucose molecules and generated electrons. If the electrons reduced the disulfide bond, insulin was freed from the membrane system. In the system depicted in Fig. 5A, enzyme co-factors NAD and FAD, which act as electron mediators, were immobilized to the membrane, but GDH was not. This membrane was referred to as a type A membrane. In the Fig. 5B system, GDH was also immobilized. This was referred to as a type B membrane. These systems were realized a sharp on-off response to the insulin release as shown in Figure 6. GDH-immobilized membrane responded to smaller amount of glucose than non-immobilized one. The immobilized GDH should have efficiently transferred the generated electrons. The membrane was specific to glucose.

ENZYME DEVICE

In order to further improve the efficiency of insulin release from the membrane device, enzyme device was synthesized, in which insulin was connected to an enzyme, GOD, with an intervening disulfide linkage as shown in Figure 7 (16,17). In contrast to GDH, GOD possesses a co-factor FAD and functions without demanding the addition of FAD.

The synthetic scheme is illustrated in Figure 8. By the esterification reaction 55% of the carboxylic groups in insulin were esterified with methanol. When the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was coupled to insulin, the elemental analysis of the reaction product of insulin and DTNB in a molar ratio of 1/5 gave the sulfur

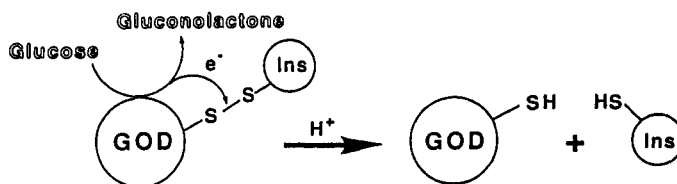


Figure 7 Insulin-releasing mechanism of the protein device in response to glucose addition, Ins representing insulin.

Table 1 Properties of Insulin/GOD hybrids

Sample	Ins/GOD in feed (mol/mol)	Urea treatment	Ins/GOD in hybrid (mol/mol)	Insulin release/ glucose addition ^a
1	10/1	+	6/1	7.5
2	3/1	+	2/1	6.0
3	3/1	-	1/1	4.5

a) The value means the molecule number of insulin released by 100 molecules of added glucose.

content of 3.36%, indicating that two of the three amino groups in a molecule of insulin were used for the reaction with DTNB. The DTNB-insulin was coupled to GOD under several conditions as shown in Table 1. The composition in the device was regulated by the feed ratio and urea treatment.

HPLC diagrams of aqueous solutions containing the insulin/GOD hybrid or unmodified insulin before and after glucose addition are shown in Figure 9. A new peak appeared on adding glucose in the

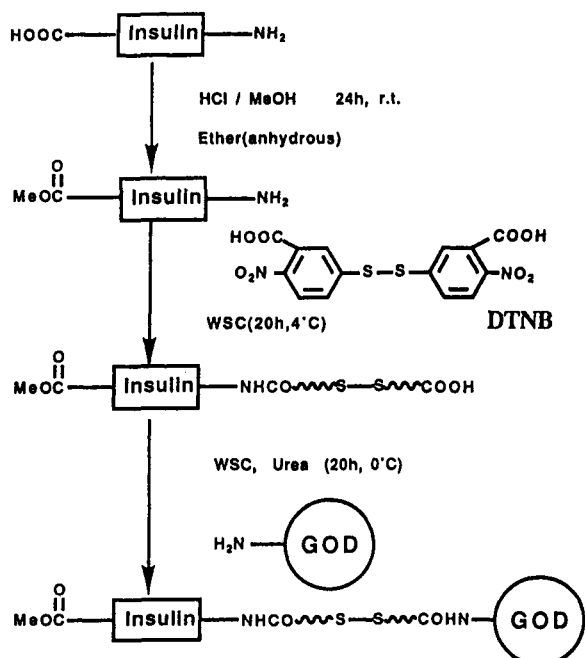


Figure 8 Synthetic scheme of the enzyme device

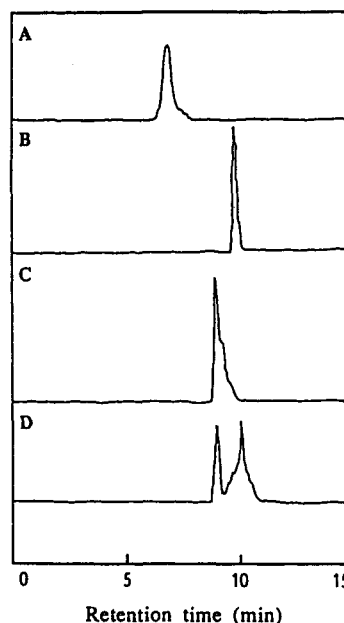


Figure 9 HPLC diagrams of unmodified GOD (A), untreated insulin (B), insulin/GOD hybrid (C), and insulin/GOD hybrid + glucose (D).

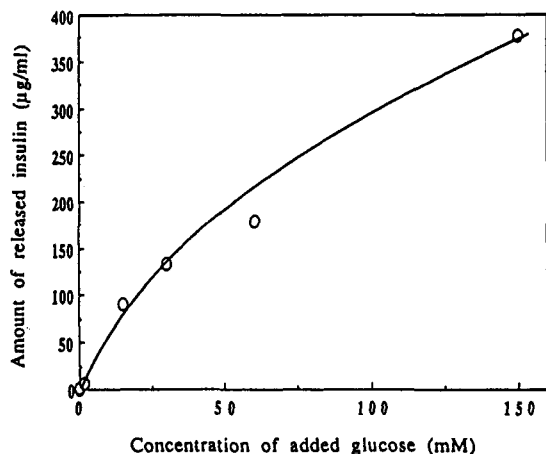


Figure 10

Release of insulin from the insulin/GOD hybrid in response to different concentrations of glucose addition.

solution containing the insulin/GOD hybrid. The retention time of the new peak almost agreed with that for the unmodified insulin, although the new peak was not completely the same as that of the native insulin since the released insulin was methyl esterified and carried 2-nitro-5-mercaptobenzoic acid. The retention times of both A and B chains of insulin were markedly longer than that of the native insulin. These observations imply that the insulin/GOD hybrid released insulin upon addition of glucose without side reactions, e.g., a cleavage of disulfide linkages of insulin itself. This conclusion was also deduced from the result that the biological activity of the released insulin was about 80% of that of unmodified insulin.

The insulin was immediately released from the hybrid on adding an aqueous glucose solution. The amount of insulin released 30 min after addition of glucose solution of varying concentrations was measured and the data are shown in Figure 10. The amount of released insulin increased with increasing concentration of glucose solution. However, the rate of insulin release was not controlled by the concentration of glucose added. It was considered that the insulin would have a maximal rate as long as the glucose concentration was higher than its K_m with GOD.

Figure 11 shows the insulin release from the several hybrids synthesized as shown in Table 1. A protein hybrid having more insulin incorporated released more insulin on addition of the same quantity of glucose. However, the efficiency of insulin release decreased with the

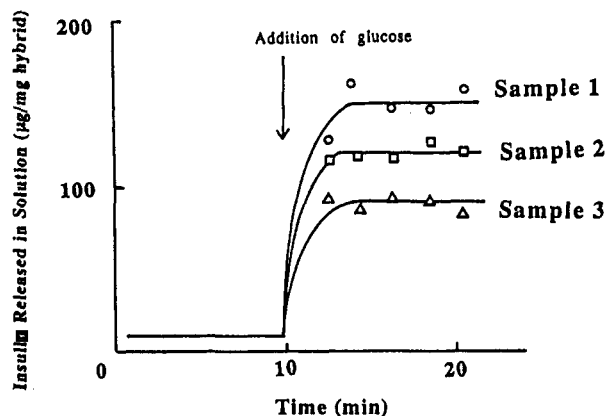


Figure 11

Insulin release from enzyme devices by addition of 2 mM glucose.

increase of insulin content in the hybrids as shown in the last column of Table 1. Samples 1 and 2 needed 3.6 and 1.5 times more glucose to cleave one disulfide linkage (to release one insulin molecule) than Sample 3, respectively. The difference should have been caused by protein denaturation. Incorporation of a large amount of macromolecular insulin into GOD should suppress renaturing of the enzyme after removing urea, although recovery was apparently observed in the incorporation of low-molecular-weight ferrocene derivative.

This enzyme device was constructed using protein and other molecules as machinery parts. This concept will be important for the design of a nano-machine. Recently not only sensing and actuating functions but also processing function is designed and being incorporated into the device (18).

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