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Development of glucose-triggered pH-sensitive liposomes for a potential insulin delivery

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Summary

pH-sensitive liposomes (pH-SL) have been studied as a glucose-responsive insulin delivery system. pH-SL were prepared using β-palmitoyl-γ-oleoyl-L-α-phosphatidylethanolamine and oleic acid. pH-dependent destabilization of the pH-SL was tested by the release of a fluorophore, aminonaphthalene-3,6,8-trisulfonic acid (ANTS) from the liposome coencapsulating a quencher, *N,N*-p-xylenebispyridinium bromide. At pH above 6.9, the release of liposomal ANTS was less than 5%, but below pH 6.4, it increased linearly with decreasing pH down to pH 5.4. The liposomes were aggregated at acidic pH, but were restored to a fine dispersion at neutral pH. To develop pH-SL as a glucose-triggered insulin delivery system, glucose oxidase (GOD) and insulin were coencapsulated in the liposomes. GOD played the role of a sensor of glucose by converting the permeated glucose to gluconic acid that led to the destabilization of the liposomal membrane releasing the content, insulin. The amount of glucose permeated into the liposome was dependent upon the external glucose concentration. Glucose permeated into the liposomes rapidly and the liposomal glucose concentration reached a maximum within 30 min. At the higher glucose concentration (900 mg/dl), the destabilization of GOD-encapsulated pH-SL was promoted, and the release of insulin was 7–8-fold enhanced compared to the lower glucose concentration (90 mg/dl). These results suggest that pH-SL coencapsulating insulin and GOD might have the potential to be developed as a glucose-triggered insulin delivery system.

Introduction

Although great improvements have been made in the effective insulin therapy of diabetes mellitus, insulin therapy still poses several problems to be overcome. One of the greatest problems is the extremely short biological half-life (3–4 min) of insulin in the circulation (Sherwin et al., 1974). To solve this problem, infusion devices have been widely developed (Kraegen and Chisholm, 1985; Selam and Charles, 1990). However, the infusion system may cause the patient discomfort, pain, and possible infection at the injection sites. Further, the prolonged hyper-insulinemia due to conventional infusion therapy can lead to adverse effects such as severe hypoglycemia (Muhlauser et al., 1985) and ketoacidosis (Bending et al.,

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1985). Therefore, recent studies have been focused on self-regulatory insulin release systems that can carry out feedback control of insulin delivery, based on blood glucose levels, using polymers such as self-regulatory insulin hydrogels (Kost et al., 1985) and microspheres (Pai et al., 1992). However, in many cases, the polymer-based delivery systems are immunogenic and non-biodegradable. Even when they are biodegradable, the cytotoxicity of the metabolites may be a problem (Smith and Hunneyball, 1986; Lherm et al., 1992).

Meanwhile, liposomes have the advantages of biodegradability and low cytotoxicity. Insulin liposomes have been studied for enhancing oral absorption and sustained insulin release (Spangler, 1990). Recently, pH-sensitive liposomes (pH-SL) have been developed to release the aqueous contents in response to a change in the external pH (Straubinger et al., 1985; Ropert et al., 1992). Most pH-SL were designed to be destabilized by the acidification of the endosome after uptake into the cells.

We developed pH-sensitive insulin liposomes with self-regulatory release properties in response to glucose by incorporating glucose oxidase (GOD). Since GOD can convert the permeated glucose in the liposomes to gluconic acid, it can lower the internal pH of liposomes and destabilize the liposomal membrane. Thus, glucose influx into the liposomes is expected to trigger the release of insulin from the liposomes (Fig. 1). In this report, the pH-dependent characteristics of pH-SL were also evaluated using a fluorophore, and the properties of GOD-containing pH-SL were studied for glucose-triggered insulin delivery.

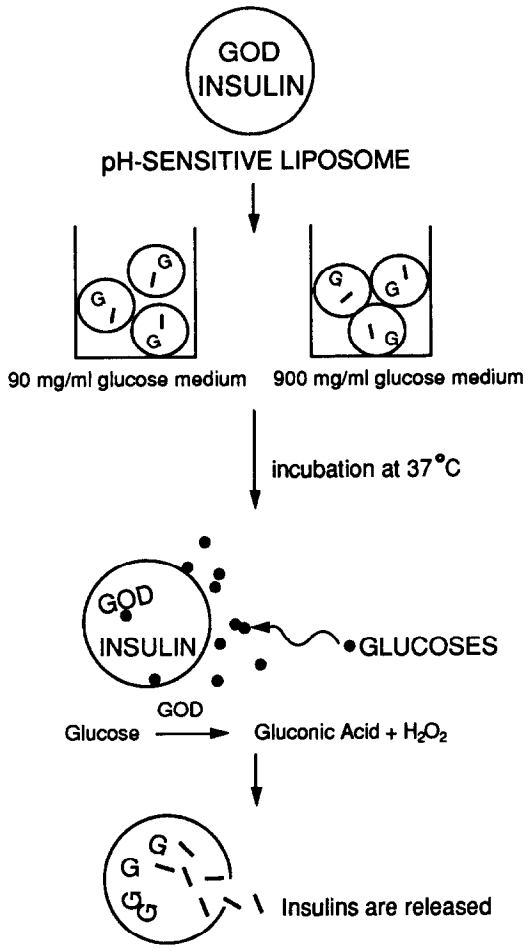

Fig. 1. Glucose-responsive insulin delivery system by pH-SL. When glucose permeates into the pH-SL containing insulin and GOD, it is converted by GOD to gluconic acid which can decrease the inside pH of liposome. The pH-SL are destabilized by the acidic pH and release the insulin.

Materials and Methods

Materials

β-Palmitoyl-γ-oleoyl-L-α-phosphatidylethanolamine (POPE), oleic acid (OA), bovine pancreas insulin (25.7 IU/mg), glucose oxidase (GOD, 151 000 IU/g), glucose and hexokinase diagnostic kits for glucose assay were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *N,N-p*-xylenebispyridinium bromide (DPX) were supplied by Molecular Probes Inc. (Eugene, OR, U.S.A.).

All other chemicals were of analytical grade and used without further purification.

Preparation of pH-SL

Large unilamellar vesicles were made by a reverse phase evaporation (REV) technique using 10 μmol of a 7:3 mixture of POPE and OA (Szoka and Papahadjopoulos, 1978). The lipids were dissolved in appropriate amount of chloro-

form and the organic solvent was evaporated on a rotary evaporator. The dried lipid film was then redissolved in 4 ml of an organic phase consisting of diethyl ether and chloroform in the volume ratio 2:1 and then 1 ml of aqueous phase was added. The organic and aqueous phases were emulsified using a bath-type sonicator for 2–4 min. The organic phase of the emulsion was then removed on a rotary evaporator for 20 min. The resultant liposomes were separated from the unencapsulated material by gel filtration on Sephadex G-50 and stored at 4°C until use.

Measurement of pH-dependent content release from pH-SL

pH-dependent release of contents from pH-SL was measured according to the procedure of Ellens et al. (1985). ANTS (12.5 mM) and DPX (45 mM) in 10 mM Tes buffer containing 65 mM NaCl at pH 7.4 were encapsulated into the liposomes. Unencapsulated materials were removed using Sephadex G-50. The eluted liposomes were resuspended in 150 mM NaCl/0.1 mM EDTA/10 mM Tes, pH 7.4 at a concentration of 0.05 mM lipid. The release of liposomal ANTS at each pH was measured by suspending liposomes containing ANTS and DPX in 10 mM iso-equiosmotic Tes buffers of various pH values at 37°C. The baseline fluorescence of the liposomes at pH 7.4 was assigned as 0% release, and the fluorescence of the liposomes completely lysed with 5% Triton X-100 was taken as 100% release. Leakage was initiated by adding 0.2 ml of the liposome suspension into a quartz cuvette containing 2.4 ml of buffer. ANTS fluorescence was measured at a wavelength of 360 nm for excitation and at 490 nm for emission using a spectrofluorometer (model 777, JASCO, Tokyo, Japan).

Measurement of pH-dependent liposomal aggregation

Aggregation of liposomes was measured based on the increase in turbidity at 450 nm using a Pye-Unicam PU 8800 UV/Vis spectrophotometer (Sowers, 1987). 200 μl of pH-SL was dispersed into 10 mM Tes buffer (pH 7.4), and the pH of the liposome suspension was then lowered to 5.4 by the addition of 0.5 N citric acid, and returned to 7.8 by the addition of 0.1 N NaOH.

Measurement of glucose permeability

To determine the amount of glucose permeated into the liposomes, two different concentrations of glucose media were employed. 200 μl of liposomal suspension was incubated with 3.8 ml of 90 or 900 mg/dl glucose media at 37°C for 4 h. At the indicated time points, aliquots were centrifuged at $100\,000 \times g$ for 30 min using a Beckman L-80 ultracentrifuge (Beckman, Palo Alto, CA, U.S.A.) to separate the glucose permeated into the liposomes from that remaining in the in suspension. Using the pellet, the amount of glucose was assayed employing the glucose hexokinase kit.

Determination of GOD in pH-SL

pH-SL containing GOD (GOD-pH-SL) were prepared by adding 1 ml of GOD in 10 mM Tes buffer at various concentrations to 10 μmol lipids dissolved in organic solvents. Liposomes were prepared by the REV technique described above. To separate unencapsulated GOD, liposomes were ultracentrifuged at $100\,000 \times g$ for 30 min and washed twice with phosphate-buffered saline (pH 7.4). The amount of GOD in the supernatant was analyzed spectrophotometrically at 500 nm using a modified Lowry method. The encapsulation efficiency was calculated from the following equation:

$$\text{Encapsulation efficiency} = [(\text{total GOD} - \text{free GOD in supernatant}) / \text{total GOD}] \times 100$$

Effects of glucose on the destabilization of pH-SL

To test the permeated glucose-mediated destabilization of liposome, liposomal aggregation was measured. 200 μl of GOD-pH-SL (2.3 μmol per ml of liposomal lipid) was mixed with 3.8 ml of 90 or 900 mg/dl glucose-containing PBS and then incubated at 37°C. For the measurement of liposomal aggregation, the turbidity was read at the

indicated time points using a Shimadzu UV spectrophotometer (Sowers, 1987).

Effect of glucose on insulin release from pH-SL coencapsulating GOD and insulin

pH-SL containing both GOD and insulin were prepared by adding 1 ml of GOD (5.5 mg/ml) and insulin (6.6 mg/ml) in PBS to 10 μmol of lipids dissolved in organic solvents using REV technique described above. To separate unencapsulated GOD and insulin, liposomes were subjected to gel column chromatoraphy on a Sephadex G-150 equilibrated with the same buffer. The unencapsulated GOD and insulin fractions were collected separately and the amounts of each compounds were respectively measured according to the modified Lowry method. The amounts of entrapped GOD and insulin were determined, respectively.

The pH-SL pellet was resuspended with PBS to adjust the concentration of pH-SL to 10 μmol of lipids/ml. Then, 200 μl of pH-SL containing GOD (2.3 μmol/ml of liposomal lipids) and insulin (2.14 μmol/ml of liposomal lipids) were suspended in 3.8 ml of PBS containing 90 or 900 mg/dl glucose and then incubated at 37°C with shaking. At the indicated time points, the released insulin was separated using Sephadex G-150 pre-equilibrated with PBS. The amount of released insulin was assayed by the modified Lowry method.

Results and Discussion

pH dependence of content release

To study the pH dependence of content release, ANTS and its quencher (DPX) were incorporated into liposomes composed of POPE and OA. ANTS fluorescence is known to be insensitive to pH between pH 4.0 and 7.5, and can be used to measure the leakage of contents from pH-SL (Ellens et al., 1984). As shown in Fig. 2, ANTS inside the intact liposomes at neutral pH is not fluorescent, since DPX is incorporated in an amount sufficient to quench all the ANTS in the liposomes. However, at acidic pH the destabilized liposomes would release both ANTS and

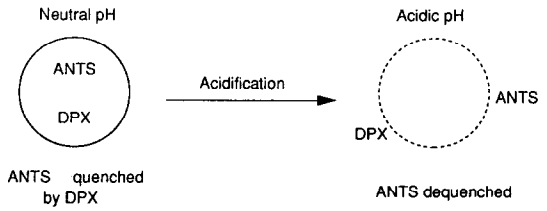

Fig. 2. The use of ANTS as a pH-SL destabilization marker. ANTS is totally quenched in the intact pH-SL by a quencher, DPX. The ANTS released from the destabilized pH-SL is dequenched, and shows increased fluorescence.

DPX. The DPX released is highly diluted in the medium and loses the ability to quench ANTS, so that the ANTS released can recover its fluorescence. Fig. 3 shows that the ANTS released from the liposomes was strongly dependent on the pH of the medium. At pH above 6.9, the extent of ANTS release was less than 5%. However, when the pH fell to 6.1, about 50% of the total liposomal ANTS was released. At pH 5.4, all the contents were released within 2 min (Fig. 4). This indicates that the pH-SL composed of POPE and OA show a pH-dependent pattern of content release in the pH range of 5.4–6.9.

Effect of pH on liposomal destabilization

The rates of liposome destabilization at acidic and neutral pH values were compared based on the time course of the turbidity change at 450 nm. Fig. 5 demonstrates that the pH-SL were destabilized immediately at pH 5.4. On raising the pH of

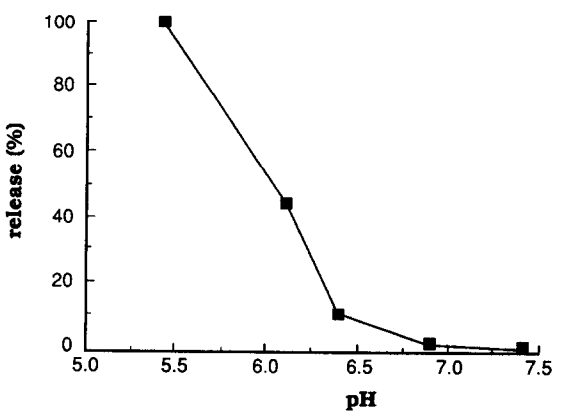

Fig. 3. pH dependence of ANTS release from pH-SL coencapsulating ANTS and DPX.

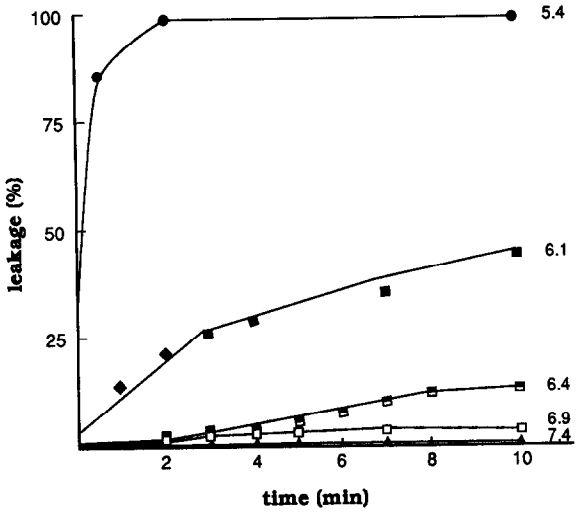

Fig. 4. Time course of ANTS release from pH-SL incubated in Tes buffer of various pH values: (●) pH 5.4, (■) pH 6.1, (□) pH 6.4, (□) pH 6.9, (▶) pH 7.4.

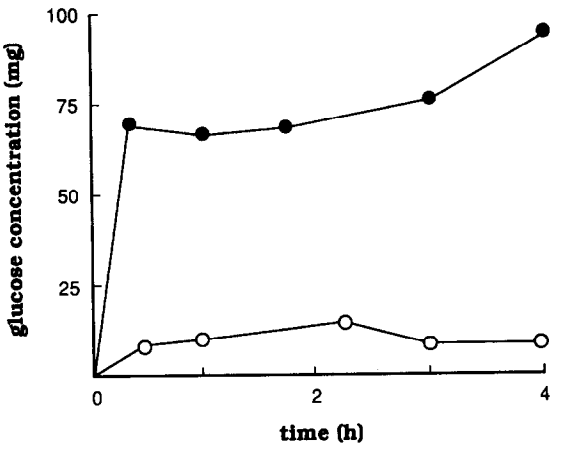

Fig. 6. Concentration of glucose permeated into the pH-SL at different concentrations of glucose medium: 90 mg/dl (○) and 900 mg/dl (●) glucose medium.

the medium to 7.8, the liposomes became rapidly disaggregated and formed a fine dispersion again. The reversible aggregation of pH-SL is consistent with previously reported results (Nir et al., 1981). The destabilization of pH-SL under acidic conditions might be explained by the neutralization of the negative polar lipid head groups of the pH-SL at acidic pH, leading to a reversible polymorphic phase transition from the bilayer to hexagonal phase that results in the aggregation of liposomes (Henry et al., 1981). These results imply that the pH-SL containing insulin may be destabilized and release insulin rapidly in acidic environments.

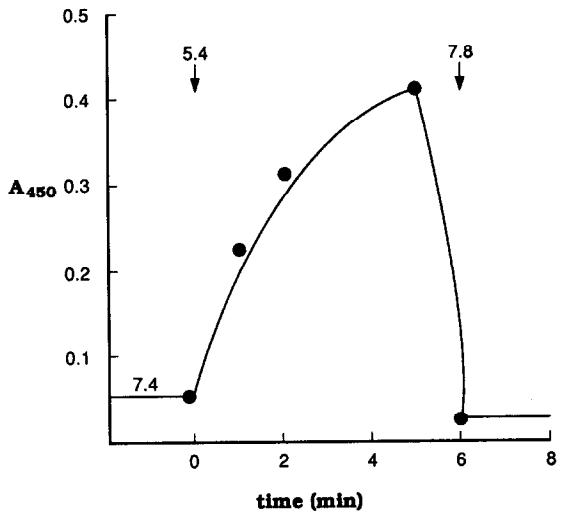

Fig. 5. Effect of pH on the destabilization of pH-SL as measured by the change in turbidity at 450 nm.

Glucose permeation into the pH-SL

Thus far, most pH-SL have been designed to release their contents on a change in the external pH of liposomes, such as the content release in acidic intracellular vesicles (Straubinger et al., 1985). However, such a large fall in pH cannot be expected to occur in the blood circulation. Thus, we designed the pH-SL to have an acidic internal microenvironment in response to external glucose by encapsulating GOD. At first, the permeation of glucose into the pH-SL was assessed in two glucose media (90 and 900 mg/dl). As shown in Fig. 6, the amount of glucose permeated in the medium of higher glucose concentration was about 7–8-fold greater than that of lower concentration. The liposomal glucose level attained the maximum value within 0.5 h after incubation, the established equilibrium being maintained over the subsequent 4 h of further incubation. Thus, glucose in the medium appears to diffuse into the pH-SL via a concentration gradient.

Efficiency of incorporation of GOD into liposomes

In order to convert all the penetrated glucose to gluconic acid efficiently, it is necessary to determine the optimum GOD concentration for use during the preparation of GOD-pH-SL. Encapsulation efficiency was improved to 42% when the concentration of GOD solution was 5.5 mg/ml (Fig. 7). Although GOD is a large macromolecule with a molecular weight of 153 000, the substantial incorporation efficiency of GOD in pH-SL may be due to the higher capture volume of REV liposomes (Szoka and Papahadjopoulos, 1978).

Effect of glucose on the destabilization of GOD-pH-SL

The destabilization of GOD-pH-SL by glucose is depicted in Fig. 8. The lower concentration of glucose (90 mg/dl) induced a slight increase in liposomal turbidity after 15 min incubation at pH 7.4. In contrast, the higher concentration of glucose (900 mg/dl) showed a rapid increase in liposomal turbidity within 15 min of incuation. The rapid destabilization of liposomes in the medium containing the higher glucose level might be due to the greater amount of gluconic acid facilitating the acidification of the liposomal internal pH. This suggests the possibility of the GOD-pH-SL functioning as a suitable glucose-responsive insulin delivery system in the blood circulation, since the pH-SL containing GOD can be destabilized by the acidification of the inside aqueous contents regardless of the outside pH.

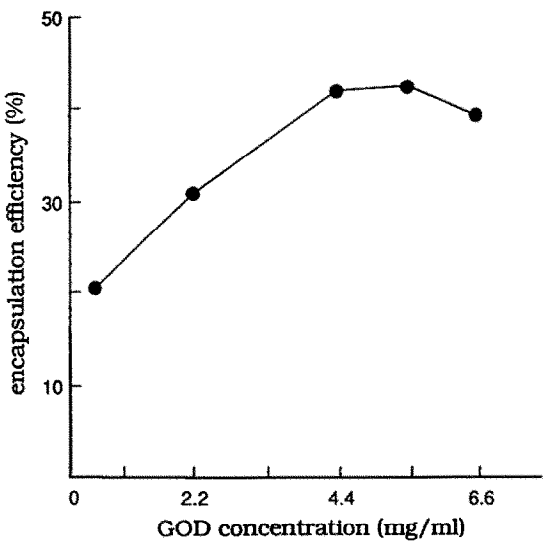

Fig. 7. Encapsulation efficiency of GOD into pH-SL at various concentrations of GOD during pH-SL preparation.

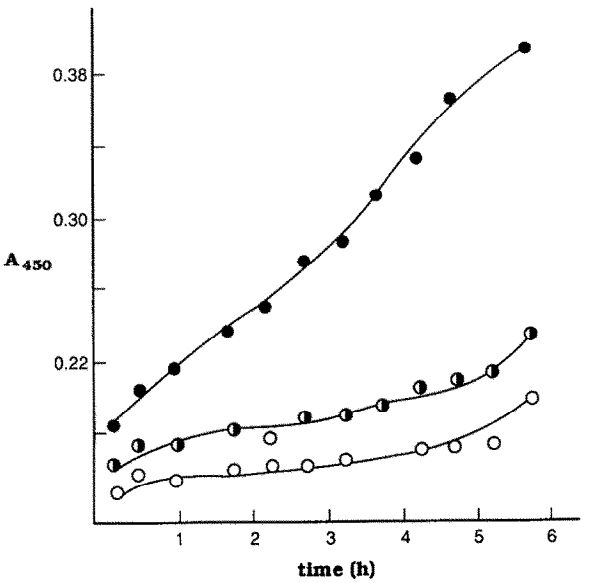

Fig. 8. Effect of external glucose on the destabilization of GOD-pH-SL as measured by the turbidity change at 450 nm. (○) Phosphate-buffered saline, (◐) 90 mg/dl glucose medium, (●) 900 mg/dl glucose medium.

Insulin release from pH-SL coencapsulating GOD and insulin by external glucose

Proper insulin therapy might be achieved by delivering an appropriate amount of insulin in response to the glucose at the required time. The amount of insulin released from the GOD-pH-SL containing insulin was shown to be governed by the external concentration of glucose (Fig. 9). The insulin released in the medium containing 900 mg/dl glucose was 7–8-fold greater than that with 90 mg/dl glucose. About 70% of the total insulin was released within 30 min. This result correlates well with the rate of permeation of glucose into the pH-SL illustrated in Fig. 6. The similarity between the rapid rate of glucose permeation and the pattern of insulin release implies that the diffusion of glucose into the liposomes might be the rate-determining step in insulin

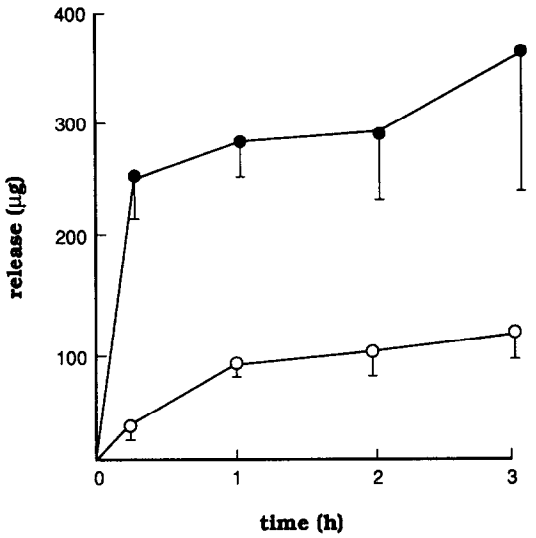

Fig. 9. Effect of external glucose concentrations on insulin release from pH-SL coencapsulating GOD and insulin. (○) 90 mg/dl glucose medium, (●) 900 mg/dl glucose medium.

release from the liposomes, and that once the glucose has permeated into the liposomes, the enzymatic reaction by GOD takes place instantaneously. The regulated and rapid release of insulin from pH-SL in response to the outside glucose level suggests that the pH-SL coencapsulating GOD and insulin may have the potential to be developed as a self-regulatory insulin delivery system. Further improvements of this system can be achieved by increasing the blood circulation time of the pH-SL with modification of the liposomal surface or lipid compositions and changing the ratio of GOD to insulin.

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