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Characterization of protein release through glucose-sensitive hydrogel membranes

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Glucose-sensitive phase-reversible hydrogels have been prepared based on the specific interaction between polymer-bound glucose and concanavalin A (Con-A). The main goal of this study was to characterize the release of model proteins (insulin and lysozyme) through the hydrogel membrane as the free glucose concentration in the environment was changed. The diffusion of the model proteins through the hydrogel membrane was examined using a diffusion cell. Porous poly(hydroxyethyl methacrylate) (PHEMA) membranes were used to sandwich the mixture of glucose-containing polymers and Con-A in between the donor and receptor chambers. The porous PHEMA membranes allowed diffusion of glucose, insulin and lysozyme, while preventing loss of glucose-containing polymers and Con-A in the sol state. The release rate of model proteins through the glucose-sensitive hydrogel membrane was dependent on the concentration of free glucose. The release rate of the proteins did not remain constant, however, due to the change in free glucose concentration resulting from diffusion of glucose from the receptor chamber to the donor chamber. This study demonstrated the possibility that the glucose-sensitive phase-reversible hydrogels can be used to regulate the insulin release as a function of the free glucose concentration in the environment.

Keywords: Drug delivery, glucose-sensitive hydrogels, sol–gel phase-reversible hydrogels, insulin release

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Conventional methods of controlled drug delivery are mainly based on diffusion through polymer membranes or matrices at a certain rate. There are many clinical situations where maintaining constant drug levels in blood is not desirable. The most widely used example is the delivery of insulin. For management of diabetes, insulin has to be released in response to the increase in the blood glucose level[1]. Daily injections of insulin are known to be inadequate for maintaining euglycaemia and preventing long-term complications of diabetes, and the demand for self-regulating insulin delivery systems is increasing. The most desirable insulin delivery system would be the one which mimics insulin release from the pancreas[2]. This requires a glucose-sensing ability and an ability to trigger release of the necessary amount of insulin[3,4].

Morris *et al.*[5] and Nakamae *et al.*[6] studied the interactions between polymer-bound glucose and concanavalin A (Con-A) and observed that such interactions resulted in precipitation of the complex. We have used a similar approach to show that polymer-bound glucose and Con-A could form a gel undergoing reversible gel–sol phase transition[7,8]. Subsequently, Taylor and Adams[9] used the same system to examine the insulin release as a function of the environment glucose concentration. Miyata *et al.*[10] also reported preparation of glucose-sensitive hydrogels using poly(2-glucosyloxyethyl methacrylate) and Con-A. In all these approaches, hydrogels are formed by specific interactions between Con-A and polymer-bound glucose molecules. Con-A with four glucose binding sites functions as a cross-linker for the glucose-containing polymer chains. Such hydrogels were found to transform to sol due to competitive desorption of polymer-bound glucose from the binding sites of Con-A by external free glucose. Sol-to-gel phase transformation occurred upon removal of free glucose by dialysis. In our previous reports[11,12], we have described the synthesis and characterization of glucose-sensitive hydrogels formed by mixing Con-A with poly(acrylamide-*co*-allyl glucose) (poly(AAm-*co*-AG)) or poly(vinylpyrrolidone-*co*-allyl glucose). In those studies we have evaluated glucose-sensing abilities of the hydrogels and the glucose-dependent gel–sol phase transition. Since the ultimate application of glucose-sensitive hydrogels is in the development of self-regulating insulin delivery systems, we examined the release of model proteins, insulin and lysozyme, through the glucose-sensitive hydrogels. The primary goal of this study was to demonstrate that the glucose-sensitive hydrogel membranes could respond (i.e. undergo reversible sol–gel phase transition) to the

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dynamic changes in external glucose concentration and that such a response could control the release of insulin through the hydrogel membrane.

MATERIALS AND METHODS

Formation of hydrogels

The synthesis of glucose-containing copolymers and formation of hydrogels was described previously[11,12]. Briefly, glucose-containing copolymer chains were synthesized by copolymerizing allyl glucose (AG) and acrylamide (AAm). Glucose was modified to AG by reacting α-D-glucose with allyl alcohol in the presence of HCl[13]. AG was copolymerized with AAm by free radical polymerization in the presence of azobisisobutyronitrile (AIBN) as the initiator at a concentration of 1% w/w of monomers. Monomers and initiator were dissolved in dimethyl sulphoxide (DMSO). Reaction mixtures were heated at 70°C for 12 h and precipitated by using methanol as non-solvent. The copolymers were filtered and dried under vacuum for 24 h at room temperature. The gels were formed by mixing the appropriate concentrations of the copolymer and Con-A in phosphate-buffered saline (PBS) containing $CaCl_2$ and $MnCl_2$ (1 mM each). The concentrations of the polymer-bound glucose and Con-A in the gel were 0.57 and 100 mg ml^{-1}, respectively.

Purification of Con-A

Con-A was purified to remove the small, fragmented chains that are known to exist within commercial preparations of this protein[14]. Con-A was dissolved in 1% ammonium bicarbonate to a final concentration of 10 mg ml^{-1}. The solution was incubated at 37°C for 15 h. The precipitate formed containing the small, fragmented chains was removed by centrifugation at 3000 rpm for 20 min. The clear supernatant was removed and dialysed extensively against distilled water. The dialysed supernatant was then lyophilized with a yield of about 50% by weight.

Preparation of porous poly(hydroxyethyl methacrylate) (PHEMA) membranes

PHEMA membranes were made by redox polymerization according to the method described by Ronel *et al.*[15]. The solution was composed of 24.75% (v/v) hydroxyethyl methacrylate (HEMA), 0.25% (v/v) ethyleneglycol dimethacrylate (EGDMA), ammonium persulphate (0.25% (w/w) of monomer), sodium metabisulphite (0.25% (w/w) of monomer) and water. Initially, the monomer and the initiator solutions were prepared and degassed separately and cooled to 5°C prior to use. The solutions were then mixed and placed between 16 cm × 18 cm glass plates with 0.2-mm-thick Teflon spacers placed between the plates to control the thickness of the resulting membranes. The solution was allowed to polymerize at 5°C for 2 h and at room temperature for 18 h. At the end of the polymerization process, the glass plates were separated and the membranes lifted carefully from the plates. The membranes were soaked in distilled water to remove any unreacted monomer and initiators. The water was changed several times and the membranes were stored in distilled water to prevent dehydration.

Protein release experiments

Release of model proteins, lysozyme and insulin, through glucose-sensitive hydrogel membranes was examined using a diffusion cell consisting of two chambers of equal volume (160 ml). Molecular weights of lysozyme and insulin are 14 400 and 5800, respectively. PHEMA membranes were used to sandwich the glucose-sensitive hydrogel in between the diffusion chambers. The PHEMA membranes were equilibrated with the buffer solution by soaking them in the buffer overnight before the experiments. Phosphate-buffered saline (PBS) solution containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$ was used throughout the study. A spacer gasket made of Teflon (0.5 mm thick) was placed between the chambers to hold and maintain the thickness of the gel. Thus, the path length through the gel was dictated by the thickness of the gasket. The surface area of the membrane available for diffusion was 12.5 cm^2. The donor chamber was filled with a protein solution in PBS and the receptor chamber was filled with PBS without any glucose. At the beginning of the diffusion experiment, a known amount of glucose was added to bring the glucose concentration in the receptor chamber to a certain level. Aliquots (1 ml each) were taken from the receptor chamber at the timed intervals to determine the concentration of the released lysozyme. The same volume of PBS was added to keep the total volume of the receptor chamber constant. Both chambers of the diffusion cell were stirred at the same speed by using magnetic stirring bars in order to minimize boundary layer effects.

For lysozyme release experiments, the donor chamber was filled with a 0.2 mg ml^{-1} lysozyme solution. The concentration of lysozyme in the sample was determined by measuring its intrinsic fluorescence by tracking its maximum emission at 340 nm after excitation at 295 nm using a fluorescence spectrophotometer (Aminco, SLM Instrument, Inc.). A calibration curve was constructed by using solutions of known concentrations of lysozyme. The lower detection limit was 0.25 μg ml^{-1}. All experiments and measurements were performed at room temperature. In all experiments involving insulin, urea was added to the PBS solution at a concentration of 2 mg ml^{-1} to minimize the aggregation of insulin during the experiments[16]. Crystalline biosynthetic human insulin was a gift from Eli Lilly and Company. Insulin was stored in a −70°C freezer until use. Insulin was first dissolved in a minimal volume of 0.1 N HCl with gentle shaking. (For example, 125 mg of insulin was dissolved in 500 μl of 0.1 N HCl.) Then, PBS was added to that solution and the pH was adjusted to 7.4 by using 0.1 N NaOH. The concentration of insulin in the stock solution was 5 mg ml^{-1}. In the diffusion experiments, the concentration of insulin in the donor chamber was 0.5 mg ml^{-1}. Insulin in the receptor side of the diffusion cell was quantitated by measuring the absorbance at 276 nm using a Beckman DU-7 spectrophotometer. The concentration of insulin was determined using a calibration curve constructed from known concentrations of insulin. According to the

calibration curve, the absorptivity of insulin at 276 nm was $1\,cm^2\,mg^{-1}$, and the lower detection limit for insulin in our study was $1\,\mu g\,ml^{-1}$. All experiments and measurements were performed at room temperature. The concentration of glucose in the diffusion chambers was measured using the phenol–sulphuric acid assay described by Dubois *et al.*[17].

RESULTS

Lysozyme release

The ability of the glucose-sensitive hydrogels to control the release of proteins as a function of the environmental glucose concentration was examined. In control experiments where glucose-free buffer was used, lysozyme was not detected in the receptor compartment during the 8 h time period. Apparently, lysozyme was not released through the membrane when the glucose-sensitive membrane was in the gel state. *Figure 1* shows release profiles of lysozyme at two different concentrations of glucose, 1 and $4\,mg\,ml^{-1}$, corresponding to normal and hyperglycaemic levels of blood glucose, respectively. At time 0, glucose was added to the receptor chamber to raise the glucose concentration to a desirable level. The glucose concentration in the receptor chamber was not maintained constant due to the diffusion of glucose to the donor chamber. When the gel was exposed to the initial glucose concentration of $1\,mg\,ml^{-1}$ in the receptor chamber, a very small amount of lysozyme was released. On the other hand, the amount of released lysozyme increased substantially when the gel was exposed to the initial glucose concentration of $4\,mg\,ml^{-1}$. In both cases, a lag time of about 30 min was observed before detecting lysozyme in the receptor chamber. *Figure 1* shows that the release of lysozyme through the glucose-sensitive hydrogel membrane was dependent on the concentration of free glucose in the environment.

In subsequent experiments, the glucose concentration in the receptor chamber was changed and its effect on the lysozyme release was examined. *Figure 2* shows the lysozyme released through the glucose-sensitive membrane while the glucose concentration in the receptor chamber was changed from 1 to $4\,mg\,ml^{-1}$ at $t = 3$ h and back to zero at $t = 8$ h. At time 0, glucose was added to the receptor chamber to raise the glucose concentration to $1\,mg\,ml^{-1}$. After approximately 30 min, the release of lysozyme into the receptor chamber was observed. At $t = 3$ h, more glucose was added to the receptor chamber to raise the glucose concentration to approximately $4\,mg\,ml^{-1}$ (arrow in *Figure 2*). After the addition of glucose, samples were collected every 10 min for the first hour to find out the response time for the increase in the release of lysozyme. As shown in *Figure 2*, the amount of released lysozyme started to increase substantially about 20 min after the increase in glucose concentration to $4\,mg\,ml^{-1}$ in the receptor chamber. The increased release of lysozyme was continued for about 30 min and started to slow down. The release rate after $t = 4$ h, however, was still larger than that observed between $t = 1$ h and $t = 3$ h. The release rate between $t = 1$ h and $t = 3$ h was $0.46\,\mu g\,ml^{-1}\,h^{-1}$, while that between $t = 4$ h and $t = 8$ h was $0.67\,\mu g\,ml^{-1}\,h^{-1}$. The release rates were calculated from the slopes of the linear portions of the release profiles. At $t = 8$ h, the entire buffer solution in the receptor side was replaced by a glucose-free buffer. Even after the glucose concentration in the receptor chamber was reduced to zero, lysozyme release was continued during $t = 9$ h and $t = 11$ h, although the released amount was very small. This is due to the glucose still remaining in the hydrogel membrane and in the donor chamber.

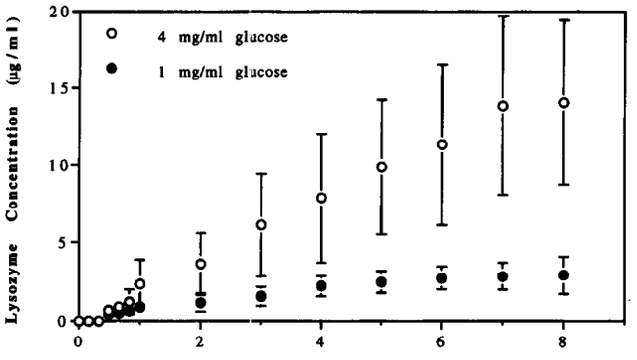

Figure 1 Release of lysozyme through a glucose-sensitive hydrogel membrane at the initial glucose concentrations of 1 and $4\,mg\,ml^{-1}$ in the receptor chamber. The lysozyme concentration in the receptor chamber was measured as a function of time. Glucose was added to the receptor chamber at $t = 0$. The concentrations of polymer-bound glucose and Con-A in the hydrogel were 0.57 and $100\,mg\,ml^{-1}$, respectively. The concentration of lysozyme in the donor side of the diffusion cell was $0.2\,mg\,ml^{-1}$ ($n = 3$).

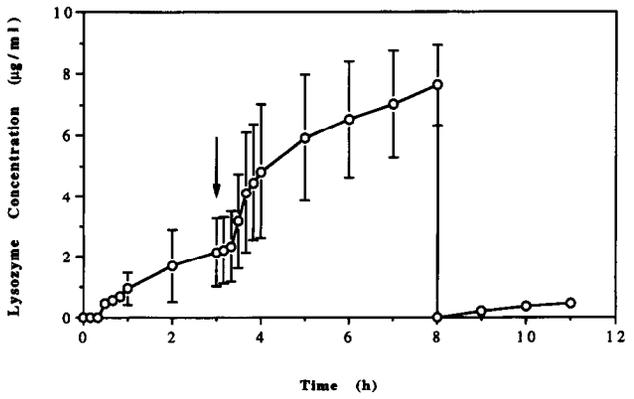

Figure 2 Release of lysozyme in response to step changes in the concentration of glucose in the receptor chamber of the diffusion cell. The lysozyme concentration in the receptor chamber was measured as a function of time. Glucose was added to the receptor chamber at $t = 0$ to make the final concentration of $1\,mg\,ml^{-1}$. More glucose was added to the receptor chamber at $t = 3$ h to raise the glucose concentration to approximately $4\,mg\,ml^{-1}$ (arrow). At $t = 8$ h the solution in the receptor chamber was replaced with glucose-free buffer. The concentrations of polymer-bound glucose and Con-A in the hydrogel were 0.57 and $100\,mg\,ml^{-1}$, respectively. The concentration of lysozyme in the donor side of the diffusion cell was $0.2\,mg\,ml^{-1}$ ($n = 3$).

Insulin release

Since the ultimate goal of our phase-reversible glucose-sensitive hydrogels is in the development of modulated insulin delivery, insulin release through the hydrogel membrane was also examined as shown in *Figure 3*. At time $t = 0$, glucose was added to the receptor chamber

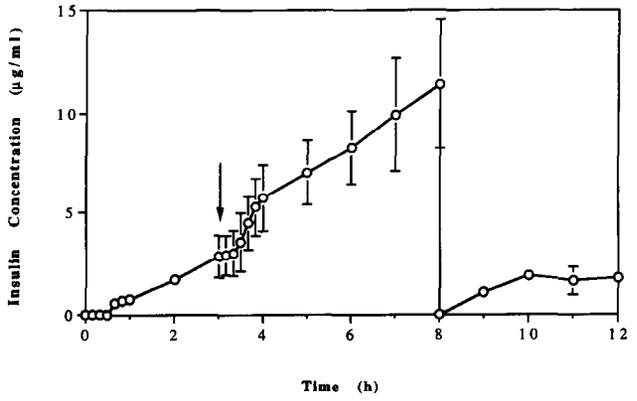

Figure 3 Release of insulin in response to step changes in the concentration of glucose in the receptor chamber of the diffusion cell. The insulin concentration in the receptor chamber was measured as a function of time. Glucose was added to the receptor chamber at $t = 0$ to make the final concentration of 1 mg ml^{-1}. More glucose was added to the receptor chamber at $t = 3$ h to raise the glucose concentration to approximately 4 mg ml^{-1} (arrow). At $t = 8$ h the solution in the receptor chamber was replaced with glucose-free buffer. The concentrations of polymer-bound glucose and Con-A in the hydrogel were 0.57 and 100 mg ml^{-1}, respectively. The concentration of insulin in the donor side of the diffusion cell was 0.5 mg ml^{-1} ($n = 3$).

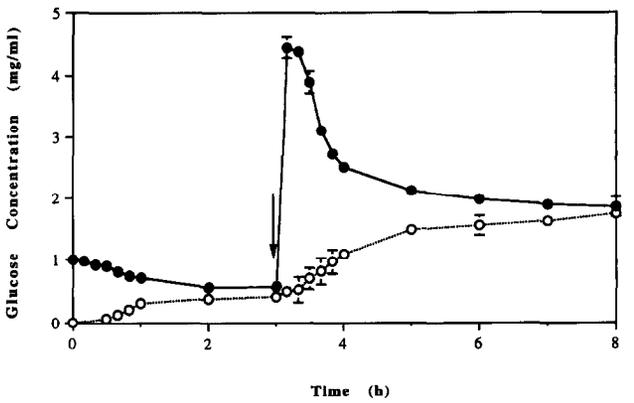

Figure 4 Changes in glucose concentration in both donor (○) and receptor (●) chambers of a diffusion cell. Glucose was added to the receptor chamber at $t = 0$ to make the final concentration of 1 mg ml^{-1}. More glucose was added to the receptor chamber at $t = 3$ h to raise the glucose concentration to approximately 4 mg ml^{-1} (arrow).

to raise the glucose concentration to 1 mg ml^{-1}. No insulin release was observed until approximately 30 min after the addition of glucose. The lag time was similar to that observed in the lysozyme release experiment, and this is quite reasonable since it should be independent of the type of protein used as long as proteins of similar molecular size are used. The release rate in the region of $t = 1$ h to $t = 3$ h was 0.98 μg ml^{-1} h^{-1}. At $t = 3$ h, more glucose was added to the receptor chamber to raise the glucose concentration to approximately 4 mg ml^{-1} (arrow in *Figure 3*). After about 20 min, the insulin release was increased significantly and the higher insulin release rate (4.62 μg ml^{-1} h^{-1}) was maintained for about 40 min. After this time (i.e. after $t = 5$ h), the insulin release was slowed down to 1.43 μg ml^{-1} h^{-1} between $t = 4$ h and $t = 8$ h. This insulin release rate was still higher than that observed before raising the glucose concentration (i.e. during time period from $t = 1$ h to $t = 3$ h). At $t = 8$ h, the whole solution in the receptor chamber was removed and replaced with buffer solution without any glucose. As shown in *Figure 3*, the insulin release was continued for another 2 h due to the glucose remaining in the hydrogel membrane and the donor chamber. At $t = 10$ h, the insulin release was stopped.

In both lysozyme and insulin release studies, the release rate increased considerably after the addition of more glucose at $t = 3$ h. The increased release rate, however, lasted only for about 40 min and the decrease in release rate after $t = 4$ h was apparent in both cases. Since such changes in the insulin release rate should be dependent on the changes in the glucose concentration in the hydrogel membrane, we examined the changes in glucose concentrations in both donor and receptor chambers of the diffusion cell. *Figure 4* shows the changes in glucose concentrations in both chambers of the diffusion cell. After more glucose was added to the receptor chamber at $t = 3$ h (arrow in *Figure 4*), the glucose concentration in the receptor chamber started to decrease, while the glucose concentration in the donor chamber started to increase. It took about 20 min for the glucose concentration in the donor chamber to increase. Since we were not able to measure the glucose concentration in the glucose-sensitive hydrogel membrane itself, we can only assume that it took about 20 min for the hydrogel membrane to transform into the sol state and thus to release more insulin. A sharp decrease in the concentration of glucose in the receptor side was observed 20 min after the addition of more glucose, with a parallel increase in the concentration of glucose in the donor chamber of the diffusion cell. At $t = 4$ h, the concentration of glucose in the receptor chamber decreased to much less than 3 mg ml^{-1}. This indicates that the glucose concentration in the glucose-sensitive hydrogel membrane was reduced, and the hydrogel became more gel state. This may be the reason why the protein release was decreased after $t = 4$ h, as observed in *Figures 2* and *3*. Since the glucose concentration was still larger than 1 mg ml^{-1}, which was the concentration of glucose at the beginning of the experiment, the protein release was still higher compared to the protein release during $t = 1$ h to $t = 3$ h. Little change in glucose concentrations in both chambers was observed after $t = 5$ h.

DISCUSSION

We have demonstrated the possibility that the glucose-sensitive phase-reversible hydrogels can be used to control the release of insulin (and lysozyme) as a function of the glucose concentration in the environment. The sol–gel phase transition of the glucose-sensitive hydrogel membrane provides not only an 'on/off' mechanism but also an ability to control the release rate of insulin. The data in *Figure 4* indicate that the lag time for glucose to appear in the donor chamber is approximately 20 min. This value is practically the same as the lag time for increase in the protein release rate after the glucose concentration in the receptor chamber was increased from 1 to 4 mg ml^{-1} at $t = 3$ h in *Figures 2* and *3*. This suggests that the sol–gel phase transition in the hydrogel

membrane is fast enough to make the glucose diffusion the rate-determining step.

For the development of self-regulating insulin delivery systems, many aspects of the system described here have to be improved. We need to develop better porous membranes that prevent the loss of the components of glucose-sensitive hydrogels in the sol state. At the beginning of our study, we tried to use a dialysis membrane to contain the gel–sol phase-reversible hydrogels. The problem was that insulin was not released through the dialysis membrane even though the molecular weight cut-off of the membrane was more than 300 000 daltons. Thus, we started using porous PHEMA membranes to contain the glucose-sensitive hydrogel during the release experiments of insulin and lysozyme. The permeability of PHEMA membranes has been studied extensively in the application of controlled and long-term delivery of insulin[15,18]. The overall good biocompatibility of PHEMA membranes for *in vivo* applications has also been established[19,20]. However, the mechanical properties of the PHEMA membranes still need to be improved, since they were found to be not strong enough to be used in implantable devices[20]. The PHEMA membranes might rupture when used in implantable devices, and this constitutes a major drawback to using such membranes *in vivo*.

Commercially available Con-A is known to contain about 50% small, fragmented chains that could diffuse through micropores of membranes[14]. Preliminary experiments using unpurified Con-A showed that about 3% of Con-A in the gel between the two PHEMA membranes was released when the gel was exposed to glucose solution (4 mg ml^{-1}). Diffusion of such small amounts was also observed by other investigators[21]. To keep the loss of Con-A to a minimum, we purified Con-A by precipitating out the small fragmented chains. Control experiments showed that no purified Con-A was released to the receptor chamber even when the glucose concentration in the receptor chamber was raised to 4 mg ml^{-1}. Prevention of the release of Con-A molecules from the glucose-sensitive hydrogel in the sol state is very important, since Con-A is an essential component to maintain the integrity of the gel and the released Con-A can cause a potential immune response if the system is used *in vivo*[22,23].

Several problems have been recognized by investigators dealing with insulin. Loss of insulin due to adsorption on surfaces is one of the most common problems encountered with insulin solution, among others such as increase in solution viscosity, precipitation from solutions and changes in physical appearance. Such problems lead to complications in the administration of insulin for the control of diabetes both in conventional administration and in the development of long-term insulin delivery systems[24]. It has been suggested that urea inhibits insulin self-association and surface adsorption by decreasing the interaction between dimers and preventing further self-association[16]. Other studies suggested the use of other stabilizers such as a copolymer of poly(propylene glycol) and poly(ethylene glycol) to prevent insulin aggregation[25]. Based on these observations, we have used 2 mg ml^{-1} of urea as additive to the buffer solutions in the diffusion experiments involving insulin. Urea, however, may not be used in clinical applications. Long-term stability of insulin in the reservoir has to be improved before any of the controlled insulin delivery devices are to be useful.

Although we have shown that the release of insulin (and lysozyme) can be controlled by adjusting the concentration of glucose in the receptor chamber, the response time may not be fast enough. In our system, the path length that insulin molecules have to diffuse through was 0.9 mm (0.5 mm by the glucose-sensitive hydrogel and 0.4 mm by two PHEMA membranes). Under such a condition, it took about 20 min to respond to the increase in glucose concentration. Furthermore, due to the diffusion of glucose to the donor chamber, the glucose concentration in the receptor chamber was not maintained constant. This resulted in changes in the insulin release rate, as shown in *Figure 3*. Reducing the thicknesses of the hydrogel and PHEMA membranes may in part solve the slow response time. The lag time of glucose diffusion through the hydrogel membrane in *Figure 4* was 20 min. The calculated characteristic time (L^2/D) for the diffusion of glucose through an unstirred boundary layer of water is also 20 min when the thickness of the water layer (L) is 0.9 mm, since the diffusion coefficient (D) of glucose in water is 6.75×10^{-6} cm^2 s^{-1}. Thus, it appears that the response time for protein release can be reduced to within 5 min if the hydrogel membrane thickness is reduced to 0.45 mm. Improvement in the response time should also consider the diffusion of glucose from the receptor to the donor chamber and vice versa. It may be difficult to control the glucose concentration in the donor chamber due to the rather easy diffusion of glucose compared to proteins such as insulin through the glucose-sensitive membranes. This implies that the control of the glucose concentration in the glucose-sensitive hydrogel membrane is quite difficult. Similar problems have been encountered by other investigators using a similar experimental set-up[9]. While it is true that many more advances have to be made before clinically useful self-regulating insulin delivery devices can be prepared, the study described in this paper presents a first step towards the development of such devices.

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