A Reversible Hydrogel Membrane for Controlling the Delivery of Macromolecules

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Abstract: Glucose-sensitive hydrogel membranes have been synthesized and characterized for their rate-of-delivery of macromolecules. The mechanism for changing this rate is based on variable displacement of the affinity interaction between dextran and concanavalin A (con A). Our main objective was to characterize the diffusion of model proteins (insulin, lysozyme, and BSA) through the membrane, in response to changes in environmental glucose concentrations. Membranes were constructed from crosslinked dextrans to which con A was coupled via a spacer arm. Changes in the porosity of the resulting hydrogel in the presence of glucose led to changes in the diffusion rate observed for a range of proteins. Gels of specified thickness were cast around to nylon gauze support (pore size, 0.1 mm) to improve mechanical strength. Diffusion of proteins through the gel membrane was determined using a twin-chamber diffusion cell with the concentrations being continuously monitored using a UV-spectrophotometer. Changes in the transport properties of the membranes in response to glucose were explored and it was found that, while 0.1*M* D-glucose caused a substantial, but saturateable, increase in the rates of diffusion of both insulin and lysozyme, controls using glycerol or L-glucose (0.1*M*) had no significant effect. Sequential addition and removal of external glucose in a stepwise manner showed that permeability changes were reversible. As expected, diffusion rates were inversely proportional to membrane thickness. A maximum increase in permeability was observed at pH 7.4 and at 37°C. The results demonstrate that this hydrogel membrane functions as a smart material allowing control of solute delivery in response to specific changes in its external environment. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **82**: 47–53, 2003.

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INTRODUCTION

In many clinical situations, controlled drug delivery systems, that deliver drugs at a predetermined rate, are replacing conventional drug formulations. However, in the case of diabetes mellitus, there is currently no entirely effective system for continuously controling blood glucose levels. The treatment of insulin-dependent diabetes requires the provision of insulin in response to increases in blood glucose concentration. Although a conventional oral route would be preferred, this is not practical for the systemic delivery of peptide and protein drugs, including insulin (Hinchcliffe and Illum, 1999). The consequent required daily injection of insulin may cause discomfort, and can lead to a number of the longterm complications associated with diabetes mellitus such as retinal damage and cardiovascular disease. Therefore, an insulin-delivery system capable of mimicking the pancreas and delivering insulin in response to increases in blood glucose would be highly attractive (Pitt, 1990).

The specific interactions between plant lectins with carbohydrates have been used to study the molecular basis of a range of biological recognition events (Loris et al., 1998; Singh et al., 1999). Such mechanisms have also been evaluated for use in drug delivery systems based on responding to chemical and biochemical stimuli, including pH, changes in specific ion concentration, and specific molecular recognition events (Qiu and Park, 2001). One approach is the development of a self-regulated insulin delivery system based on glucose-sensitive hydrogels capable of regulating the delivery of insulin in response to changes in blood glucose levels. Several such systems have been developed (Galaev and Mattiason, 1999; Qiu and Park, 2001), including pH-sensitive membrane systems, and two con-A based systems.

In the first approach, immobilized con A is used to control the delivery of a glycosylated insulin, which is initially bound by the con A. Interaction of environmental glucose

with the glycosylated insulin-con A complex leads to the displacement of glycosylated insulin (Brownlee and Cerami, 1979). A more recent approach has been the use of con-A/dextran mixtures to form a reversible sol-gel system (Obaidat and Park, 1997; Taylor et al., 1995; Tanna et al., 1999). In this system, a glucose-based polymer is mixed with con A to form a hydrogel, which is sandwiched between two porous membranes (Obaidat and Park, 1997; Tanna et al., 1999; Taylor et al., 1995). Gelation is reversible in response to changes in environmental glucose concentration such that the diffusion coefficient for insulin changes with degree of gelation. In earlier work, the gels were based solely on soluble components. However, leakage of con A through the membrane during del-sol transition led to revised systems with con A coupled to the polymer.

While reducing leakage, coupling of con A to the polymer still results in a system that is in a soluble form in the presence of glucose (Kim and Park, 2001; Tanna et al., 1999). Thus, support membranes are still required, leading to increased complexity and slow diffusion rates. To overcome these problems we report the synthesis of a mechanically stable, glucose responsive hydrogel membrane, which can be cast in a number of mechanical forms.

Our polymer is produced by crosslinking two dextrans with different molecular weights. The smaller dextran is functionalized with covalently grafted conA. This material is mixed with the larger, nonfunctionalized dextran, allowing easy control of the overall amount of grafted conA in the material. The affinity of ConA for dextran provides additional affinity crosslinks which are competitively inhibited by free glucose, resulting in a decrease in total crosslink density and increase in permeability to protein.

This is conceptually similar to the work of Miyata et al. (1999), who describe an antibody/antigen-based gel that swells in response to the free antigen. However, the use of the two dextran species allows greater control over the gel structure such that property changes can be restricted to changes in internal porosity. In this case the porosity increases because of the displacement of con-A/dextran interactions by the stimulus of soluble D-glucose.

To achieve this goal, gels were formed via a nucleophilic replacement reaction in a triazine ring, such that both gel formation and con-A fixation occurred in the same step of synthesis. A specific requirement of gel synthesis was to maintain the reaction pH below 9 to minimize con-A inactivation during coupling. In the results presented here, we demonstrate a material that is mechanically stable, changes permeability in response to changes in glucose concentration, and that shows negligible con-A leakage over extended periods.

MATERIALS AND METHODS

Materials

Dextran 40,000, dextran 500,000, divinyl sulfone, ethylenediamine, cyanuric chloride, concanavalin A (type IV from the jack bean), insulin, lysozyme, and BSA were obtained from Sigma-Aldrich, UK. All other chemicals were of reagent grade and obtained from BDH chemicals Ltd., UK.

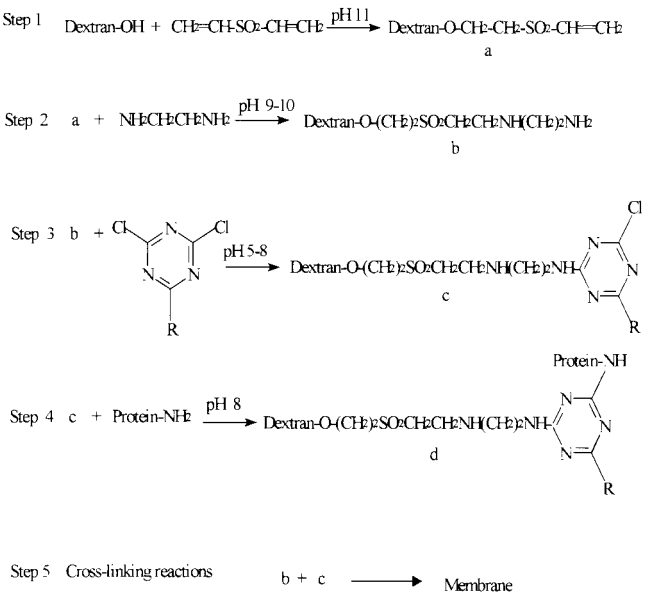


Figure 1. Synthetic route for the synthesis of concanavalin.

Smart Membrane Synthesis

The chemistry of hydrogel synthesis is shown in Figure 1. The procedure was as follows:

1. 10 g dextran was dissolved in 150 mL water, 0.795 g Na_2CO_3 was added, followed by the addition of 1.2 mL 97% divinyl sulfone. This was allowed to react at 18°C for 60 min, prior to the addition of 2.74 g ethylenediamine. The reaction was then allowed to proceed for 48 h after which the reaction mixture was dialyzed extensively against distilled water. The final dextran derivative solution was freeze-dried and then redissolved in distilled water to give a 14% dextran solution.
2. 20 mL solution of the dextran derivative was mixed with 0.17 g cyanuric chloride and allowed to react at room temperature for 24 h. The solution was centrifuged for 20 min at 4000 rpm at 4°C, and the supernatant was recovered. (The dextran derivative made with this procedure using a 40,000 mw dextran will be referred to as Solution A; the dextran derivative made using 500,000 mw dextran will be referred to as Solution B.)
3. 0.8 g of Solution A, 0.8 g of Solution B, 0.5 mL 0.8*M* $NaHCO_3$ and 40 mg con A (in 1.2 mL water) containing 1 mg $CaCl_2$ and 1.2 mg $MnCl_2$ were added and mixed fully. After mixing for 10 min, the solution was degassed and cast on a nylon gauze support (pore size 0.1 mm, thickness of 0.05 mm) between two glass plates using spacers to give the required total membrane thickness.

Gelation was allowed to proceed at 25°C for 16 h before the membrane was removed from the casting plates. The membrane was washed twice in distilled water and immersed overnight in 10 m*M* glycine solution in 20 m*M* acetate buffer, pH 6.0, containing 50 m*M* NaCl and 0.1% NaN_3 at 4°C. Finally, the gel was washed with distilled water twice and stored in 20 m*M* Tris buffer, pH 7.4, containing 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$, 50 m*M* NaCl, and 0.1% NaN_3, until it was used.

Protein Diffusion Experiments

The diffusion of model proteins (including insulin: mw 5800, lysozyme: mw 12400, and BSA: mw 66000) through the gel membranes was examined in a diffusion cell consisting of donor and receptor chambers with equal volume (4.4 mL), and with a surface area of 4.6 cm^2 available for diffusion. The gel membrane was mounted between the two chambers of the diffusion cell, and equilibrated with 20 m*M* Tris storage buffer as above. The donor chamber was connected to a protein reservoir through a pump. The receptor chamber was connected via a closed loop recycle through a flow cell mounted in the spectrophotometer (Shimadzu, UV-1601). The diffusion of the protein was continuously measured from absorbance changes at 280 nm logged to a computer. The effect of putative transport-enhancing agents was investigated by varying the composition of the donor buffer such that protein concentration remained constant.

For the experiments with insulin, the protein (from Bovine Pancreas, Sigma) was dissolved in a small amount of 0.05 N HCl and then adjusted at pH 7.4 with 0.05 N NaOH, and finally diluted with Tris buffer to give an insulin concentration of 2.0 mg/mL. In all experiments using insulin, urea was added to a final concentration of 2.0 mg/mL to inhibit insulin aggregation (Sato et al., 1983). Concentration-time courses were determined from absorbance changes using a conversion factor determined from a standard curve constructed using known insulin concentrations. For experiments with lysozyme and BSA, proteins were dissolved directly in Tris buffer to a concentration of 2.0 mg/mL. In each case the concentrations observed in the diffusion experiments were determined using calibration data from standard curves prepared using the specific protein.

RESULTS

Specificity Assessment

The specificity of the hydrogel membrane was determined using D-glucose, L-glucose, and glycerol. While D-glucose is specifically bound by con A, L-glucose and glycerol do not interact with it. Hence, membrane permeability changes would be expected in response to D-glucose, but not L-glucose or glycerol. Figure 2 shows the diffusion curves of insulin in response to glucose, L-glucose, or glycerol through a 0.4 mm membrane. In control experiments, where insulin solution without carbohydrate/glycerol was used little diffusion was observed. When L-glucose or glycerol was added to the protein reservoir of the donor side (final concentration 0.1*M*), there was a small increase in the diffusion rate in the initial stages, after which the profile mirrored that observed for the control. After a stabilizing period of 42 min D-glucose (0.1*M*) was added. Then, after a lag of about a further 18 min, there was a substantial increase in diffusion rate which was maintained for an extended period. The lag between addition and response matches the residence time in the donor cell such that diffusion limitations will be partially masked by the time course of D-glucose increase in the liquid phase.

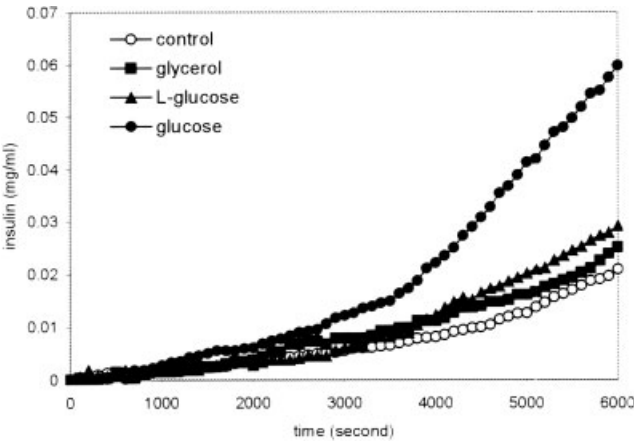


Figure 2. Insulin (2 mg/mL) diffusion curves in response to 0.1*M* D-glucose (●). L-glucose (■), or glycerol (▲). The control was carried out with insulin only (○). Putative activators were added at 2500 s. Measurements were conducted at 25°C.

The results show that the hydrogel membrane allows protein transport at a rate that is controllable using a soluble effector molecule, and that this effect is limited to molecules recognized by the con A receptor. This suggests that, as anticipated, changes in porosity of the membrane where dextran serves as both a ligand for con A affinity and a structural matrix results from a reduction of internal affinity crosslinks when a soluble competitor is introduced. It is also implicit in this result that a significant fraction of the total con A is not inactivated during membrane synthesis.

Effect of D-Glucose Concentration

Three proteins of different molecular sizes, insulin, lysozyme, and BSA, were used to examine the effect of glucose concentration on diffusion rates. Figure 3 shows the diffusion of insulin in response to changes in glucose concentrations over the range 0–200 mM. In these experiments, membranes were pre-incubated with protein (with or without glucose) in the donor side of the cell to minimize subsequent lag times. The diffusion rate obtained as a function of glucose concentration are shown in Figure 3.

These experiments were repeated with two progressively

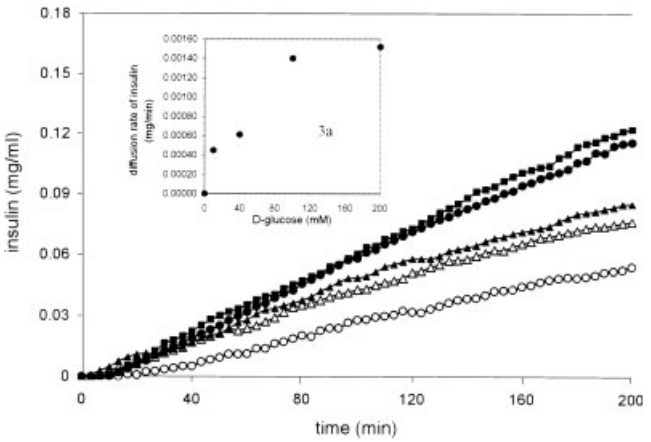


Figure 3. Insulin (2 mg/mL) diffusion in response to D-glucose concentration: 0 m*M* (control, ○), 10 m*M* (△), 40 m*M* (▲), 100 m*M* (●) or 200 m*M* (■). Glucose was added to the donor reservoir. Temperature 25°C. (a) The rate of insulin diffusion as a function of D-glucose concentration.

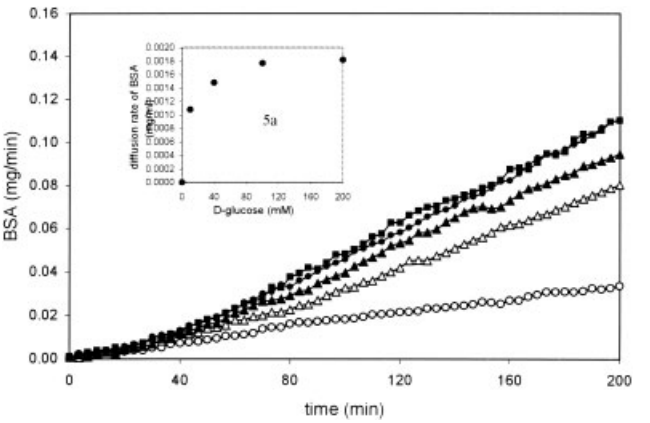


Figure 5. BSA (2.0 mg/mL) diffusion in response to D-glucose. Symbols as for Figure 5. Glucose was added to the donor reservoir. Temperature 25°C. (a) The rate of BSA diffusion as a function of glucose concentration.

larger proteins. Studies with lysozyme gave essentially similar results (Fig. 4). As might be expected from the higher molecular weight the diffusion in the absence of glucose was lower than that observed for insulin. However, glucose has a more pronounced effect on diffusion rates at low concentrations and shows a lower saturation value than that observed with insulin (Fig. 4). Results obtained with BSA are shown in Figure 5. Again, diffusion is sensitive to changes in glucose concentration with 100 m*M* glucose leading to a 3.5-fold increase in the rate of BSA diffusion. BSA diffusion in response to glucose is shown in Figure 5.

Reversibility of Glucose Effects

A series of experiments was conducted with each of the test proteins in which two reservoir solutions were used alternately: protein, and then protein containing 0.1*M* glucose. Results for insulin are shown in Figure 6. The donor solution was sequentially switched between glucose-containing and non-glucose-containing solutions as indicated by arrows on the plot. The response shows that, while there is a diffusion time lag between each solution change, the introduction and removal of glucose causes a reversible switch between lower and higher diffusion rates. Similar results obtained for lysozyme are shown in Figure 7, and for BSA in Figure 8. Again, the reversibility of the glucose effect on diffusion rate is clearly demonstrated.

Temperature, pH, and Membrane Thickness Effects

Diffusion experiments were conducted using a 0.4 mm membrane at 5°, 25°, and 37°C using lysozyme as the test

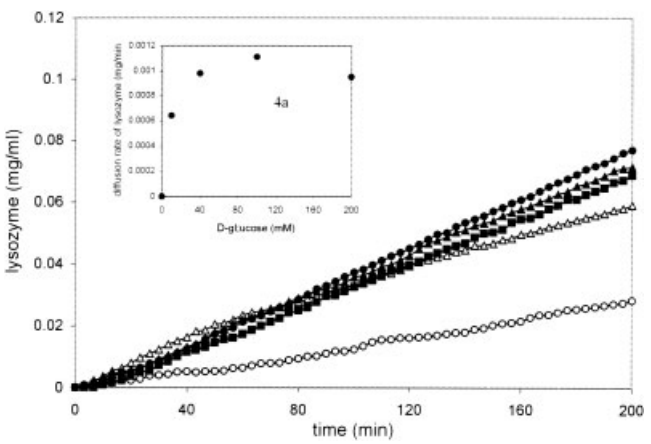


Figure 4. Lysozyme (2 mg/mL) diffusion in response to D-glucose. Symbols as for Figure 3. Glucose was added to the donor reservoir. Temperature 25°C. (a) The rate of lysozyme diffusion as a function of glucose concentration.

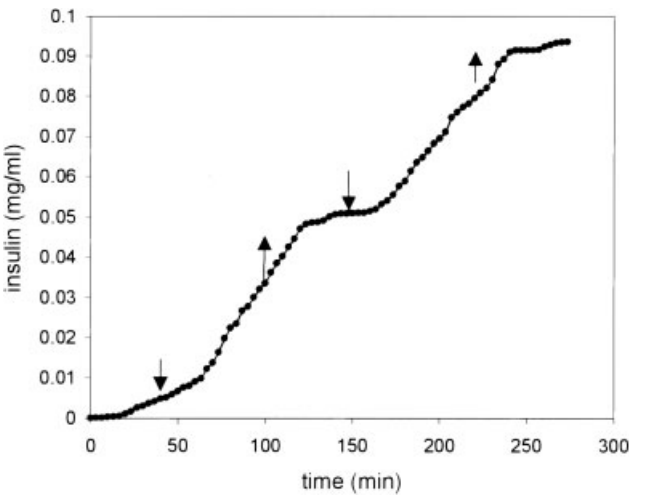


Figure 6. Reversibility of D-glucose effects on insulin diffusion. 0.1*M* glucose plus insulin (2.0 mg/mL) was added at (down-arrows): 42 min and 150 min. Insulin solutions without glucose were added at (up-arrows): 100 min and 225 min. Temperature 25°C.

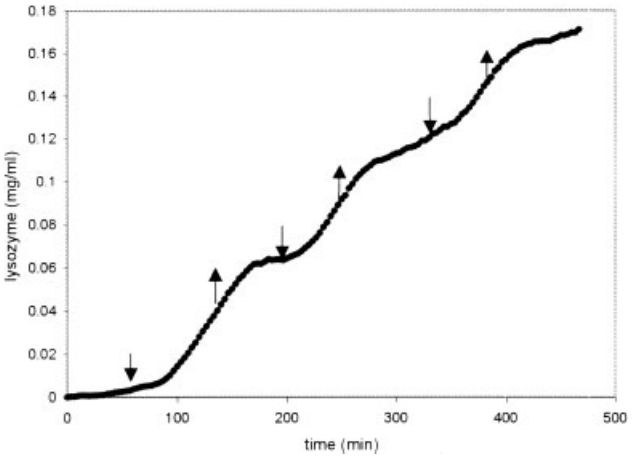


Figure 7. Reversibility of D-glucose effects on lysozyme diffusion. 0.1*M* glucose plus insulin (2.0 mg/mL) was added at (down-arrows): 65 min, 200 min, and 335 min. Lysozyme solutions without glucose were added at (up-arrows) 140 min, 250 min, and 383 min. Temperature 25°C.

protein. Glucose was introduced after 100 min. Results shown in Figure 9 confirm that diffusion rates increase with temperature between 5° and 25°, but that little additional increase is observed between 25–37°. It would appear that there is a slightly greater effect on specific transport compared with the nonspecific component.

Experiments with different gel thickness (Fig. 9b) show that diffusion rate decreases with increasing membrane thickness, as expected from Fick's law. More importantly, the fractional increase in diffusion rate obtained when glucose is introduced appears inversely proportional to membrane thickness.

Figure 9c shows pH effects over the range 5.4–8.4. This shows an optimum response at pH 7.4.

DISCUSSION

In undertaking this project, our goal was to produce a chemically and mechanically stable membrane capable of

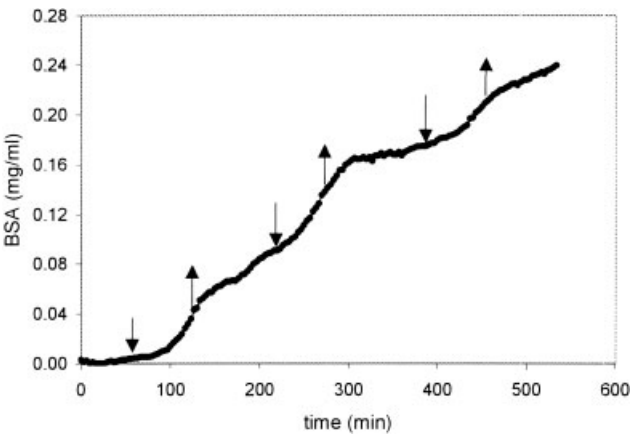
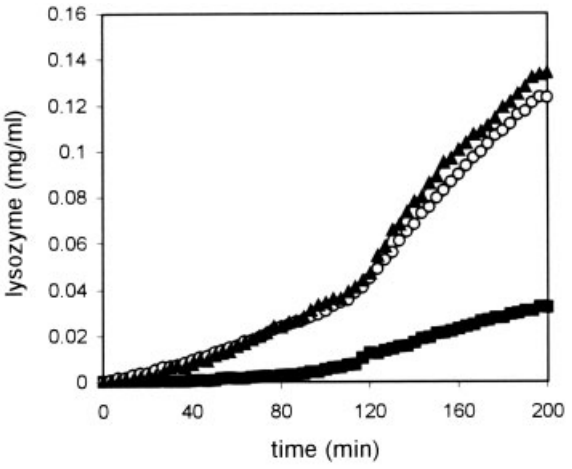
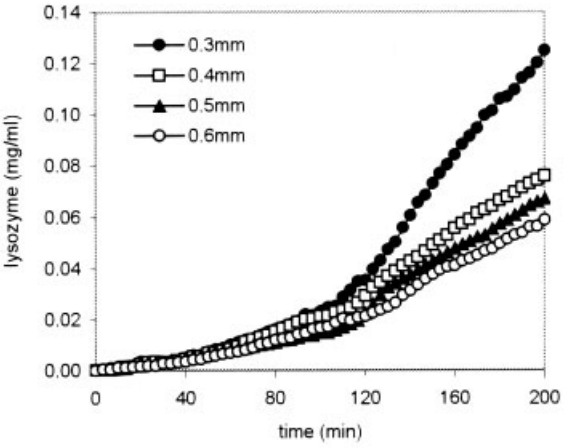


Figure 8. Reversibility of D-glucose effects on BSA diffusion. 0.1*M* glucose plus BSA (2.0 mg/mL) was added at (down-arrows): 67 min, 222 min, and 385 min. BSA solutions without glucose were added at (up-arrows): 125 min, 283 min, and 467 min. Temperature 25°C.



(a)



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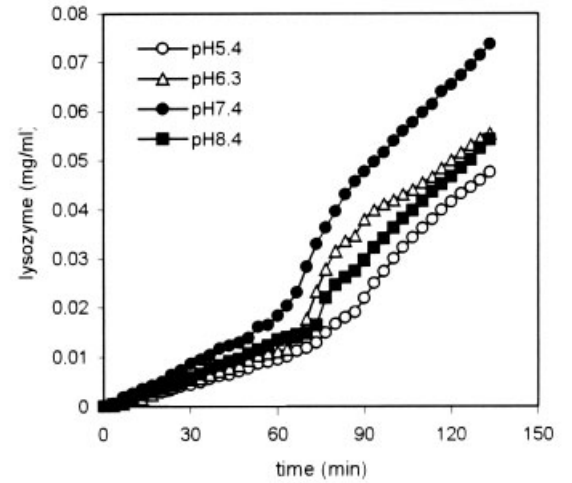

(c)

Figure 9. (a) Effects of temperature on the diffusion of lysozyme (2.0 mg/mL). 0.1*M* D-glucose was added to the donor reservoir 100 min. Temperatures 5°C (■), 25°C (○), and 37°C (▲). (b) Effects of membrane thickness on the diffusion rate of lysozyme (2 mg/mL). D-glucose (0.1*M*) was added to the donor reservoir at 100 min. Temperature 25°C. (c) Effects of pH on the diffusion rate of lysozyme (2 mg/mL). D-glucose (0.1*M*) was added to the donor reservoir at 50 min. Temperature 25°C.

specific permeability changes in response to concentration changes in external metabolites. While generic in scope, the initial application envisaged was the controlled release of insulin in response to D-glucose concentration.

Initial experiments to assess the mechanical stability of pre-cast membranes showed no evidence of dissolution over a 1-week incubation period in storage buffer. Assays of buffer composition showed no evidence of con A leakage during this period.

The first phase of characterization studies aimed to establish: (1) that permeability as evidenced by protein diffusion, could change in response to concentration changes in D-glucose concentration, and (2) that this effect was specific. The results presented in Figure 2 confirm that this is the case. While it is apparent that the porosity of the gel does not totally exclude insulin, the addition of D-glucose results in a threefold increase in diffusion rate. While there is a very slight increase observed with the addition of L-glucose and glycerol this probably results from a nonspecific chemical effect modifying the degree of gel swelling.

Similar experiments with a hydrogel membrane synthesized with BSA instead of con A failed to show any significant increase in diffusion when glucose was applied. This confirms that changes in the porosity of the gel membrane resulting from the dissociation of affinity crosslinks led to changes in protein diffusion. Previous reports (Ballerstadt and Schultz, 1998; Miyata et al., 1999), suggest the diffusion of glucose through hydrogels will be slower than the dissociation of con A/dextran affinity crosslinks. As proteins have lower diffusivities than sugars, the response will be determined by the diffusivity of the protein in the dissociated gel membrane. Similar specific increases in diffusion rate were observed with mannose, which is also known to bind to con A.

The effect of D-glucose concentration on the diffusion of three different-sized proteins is shown in Figures 3, 4, and 5. The inset figures show transport rate as a function of D-glucose concentration, and indicate a half-saturation constant between 40–60 m*M*. This is consistent with the displacement of a weak affinity interaction. As the size of the protein increases, the ratio of competitor induced to background diffusion also increases. This suggests that, in addition to optimizing the base level of affinity plus covalent crosslinks to ensure zero background diffusion, porosity should also be optimized with respect to maximizing competitor-induced transport.

As our goal is to make a responsive material suitable for drug delivery applications, reversibility of the selective response is an essential requirement. The results reported in Figures 6, 7, and 8, where D-glucose was sequentially added and removed, clearly show that the con-A/dextran membrane meets this requirement. Reversible responses were found with all three proteins tested.

The affinity displacement of an interaction between con A and an immobilized ligand is clearly attractive for an insulin delivery application, but it also has the specific advantage that the displaced ligand also forms part of the gel matrix. However, in the two-component synthesis protocol described here, the larger dextran might be regarded as acting as the matrix and the smaller, pendant, dextran as the ligand. In an analogous system, Miyata et al. (1999) report the synthesis of an antigen–antibody hydrogel membrane where goat anti-rabbit IgG and rabbit IgG were coupled to *N*-succinimidylacrylate separately, and then co-polymerized with acrylamide to form a hydrogel. This gel also showed a reversible response to the environmental addition of rabbit lgG, suggesting that it should be possible to develop responsive polymers for a range of solute delivery applications.

CONCLUSIONS

Dextran/con-A hydrogel membranes can be synthesized that are capable of releasing insulin in a dose-dependent response to changes in D-glucose concentrations. The response observed is selective to sugars that are bound by con A and is repeatably reversible. Data shown in Figures 6, 7, and 8 suggest a lag time of approximately 40 minutes between point of insulin introduction and increase in transport rate. While this could be improved if thinner membranes were used, it compares favorably with the 30–120 minutes reported by Lembert et al. (2001) for insulin release by free-floating islets. Unlike sol-gel systems, the membrane described here retains structural integrity in the presence of elevated D-glucose concentrations and needs no additional external membrane containment. The synthesis protocol we report can be extended to allow the attachment of ligands as well as receptors to an inert support. This would allow the formation of responsive gels based on a range of ligand-receptor interactions.

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