NAD-Sensitive Hydrogel for the Release of Macromolecules

M. Tang,[1] R. Zhang,[1] A. Bowyer,[2] R. Eisenthal,[3] J. Hubble[1]

[1]Department of Chemical Engineering, University of Bath, UK; telephone: 044 1225 386221; fax: 044 1225 386894; e-mail: j.hubble@bath.ac.uk
[2]Department of Mechanical Engineering, University of Bath, UK
[3]Department of Biology and Biochemistry, University of Bath, UK

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Abstract: A hydrogel membrane containing immobilized ligands and receptors was synthesized and investigated for the controlled diffusion of test proteins (cytochrome C and hemoglobin). Both Cibacron blue (ligand) and lysozyme (receptor) were covalently linked to dextran molecules that were subsequently crosslinked to form a gel. The resulting stable hydrogels contained both covalent and affinity crosslinks such that their intrinsic porosities were sensitive to competitive displacers of the affinity interaction between lysozyme and Cibacron blue. Transport experiments in a twin chamber diffusion cell showed that as NAD was added to the donor side, the dissociation of the binding sites between the Cibacron blue and the lysozyme led to an increase in protein diffusion through the hydrogel. The results showed that addition of NAD caused a saturable concentration-dependent increase in the transport of both cytochrome C and hemoglobin. This effect was shown to be both specific and reversible. © 2004 Wiley Periodicals, Inc.
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INTRODUCTION

Recent developments in biotechnology and genetic engineering have meant that more and more bioactive peptides and proteins have been obtained for novel drug applications (Kikuchi and Okana, 2002). However, these bioactive molecules are often not suitable for oral delivery and are usually metabolized quickly in the body. Therefore, there is a requirement for effective delivery systems if these materials are to form the basis of successful therapies.

Conventional controlled drug delivery systems are based on constant slow-release mechanisms. These materials do not respond to metabolite changes in the patient's body in order to deliver the drug at an appropriate rate; i.e., there is no feedback to improve control. Smart drug delivery systems that do respond to metabolite changes offer the potential for developing much more effective dosing regimes that mimic natural functions. For example, insulin release from the pancreas is induced by an increase of blood glucose concentration (Pitt, 1990). Systems incorporating the functions of both biosensor and actuator are required to provide complete selective feedback-controlled drug delivery (Kikuchi and Okana, 2002; Qiu and Park, 2001; Miyata et al., 2002).

Hydrogels have been extensively used in attempts to develop smart drug delivery systems. The responses used include: pH-sensitivity (Kou et al., 1988; Akala et al., 1998), temperature-sensitivity (Dong and Hoffman, 1990; Bromberg and Ron, 1998), and glucose-sensitivity (Brownlee and Cerami, 1979; Ito et al., 1989; Kitano et al., 1992; Taylor et al., 1995; Lee and Park, 1996; Miyata et al., 1996; Obaidat and Park, 1997; Hassan et al., 1997; Tanna et al., 1999; Kim and Park, 2001).

In model systems using glucose-sensitive hydrogels, specific recognition based on lectin-carbohydrate interactions has been the most widely studied (Taylor et al., 1995; Lee and Park, 1996; Miyata et al., 1996; Kim and Park, 2001). In addition to the potential application for use in the treatment of diabetes, these systems have the advantage that the structural polymer component of the gel (dextran) also acts as the ligand for the receptor (concanavalin A).

To broaden the potential application base for responsive hydrogels it is necessary to develop systems where both ligand and receptor are specifically coupled to an inert support. Miyata et al. (1999) reported the synthesis of an antigen-antibody hydrogel membrane where goat antirabbit IgG and rabbit IgG were coupled to N-succinimidylacrylate separately and then copolymerized with acrylamide to form a hydrogel. This gel showed a reversible response to the environmental addition of rabbit IgG, suggesting that it should be possible to develop responsive polymers for a range of solute delivery applications.

Here we investigate such a system aimed at producing a gel that responds to changes in concentrations of nico-

Correspondence to: J. Hubble
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tinamide adenine dinucleotide (NAD). NAD is an important biomolecule with a central role in oxidation and reduction reactions in metabolic processes (Berg et al., 2002). However, the major purpose of this study is to demonstrate the generality of our approach, which is to construct a responsive membrane by incorporating into the polymeric structure a pendant ligand and pendant receptor in such a manner that the permeability properties of the membrane may be altered in the presence of a competitor of the receptor/ligand interaction within the polymeric structure.

In this study we used Cibacron blue as the ligand and lysozyme as the receptor. Cibacron blue has a molecular shape similar to that of NAD and has been widely used as a biomimetic ligand in affinity chromatography of dehydrogenase enzymes. It also binds specifically and with high affinity to lysozyme (Mayes et al., 1990). There are also reports that lysozyme has a binding affinity for NAD (Yu et al., 2001). Considerations of cost, stability, and availability thus make the Cibacron blue/lysozyme pair a target for an NAD-responsive material.

We previously synthesized a glucose-responsive membrane where a change in the porosity of the membrane led to increased transport rates of a number of proteins of different sizes (insulin, lysozyme, and bovine serum albumin; Tang et al., 2002). In the work described here, Cibacron blue was covalently linked to a dextran chain, then a spacer arm was introduced to link lysozyme and to crosslink dextran chains to form the hydrogel. The primary target was to demonstrate that NAD-sensitive hydrogel membranes can be produced that change their porosity in response to changes in environmental NAD concentration, and that this response can control the delivery of macromolecules though the membrane.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma Chemicals (Poole, Dorset, UK) unless otherwise stated.

Preparation of Cibacron Blue–Dextran Derivatives

The NAD-sensitive hydrogel membrane synthesis protocol is shown in Figure 1.

Conjugation of Cibacron Blue (CB) to Dextran

This was based on the methodology described by Dean et al. (1985). Five grams of Dextran 500 kDa was dissolved in 100 ml water, followed by the addition of solid Na_2CO_3 (0.1 M final). CB (55% dye content) was added in five separate aliquots (total addition 4.3 g) over 24 h at room temperature. The solution was then maintained at 40°C for 24 h to form a dextran–Cibacron blue conjugate (CB–Dex). The conjugate was precipitated with ethanol (50% v/v) and redissolved in distilled water; this procedure was repeated twice to remove most of the unreacted dye. The conjugate was then extensively dialyzed against distilled water and finally freeze-dried.

Coupling of CB–Dex to Divinyl Sulfone (DVS)

This was based on the method of Porath et al. (1975); 4.0 g CB–Dex was dissolved in 40 ml distilled water and solid Na_2CO_3 was added to a final concentration of 0.1 M and 0.46 ml 97% DVS added slowly with continuous stirring. The reaction was allowed to proceed at 18°C for 80 min,

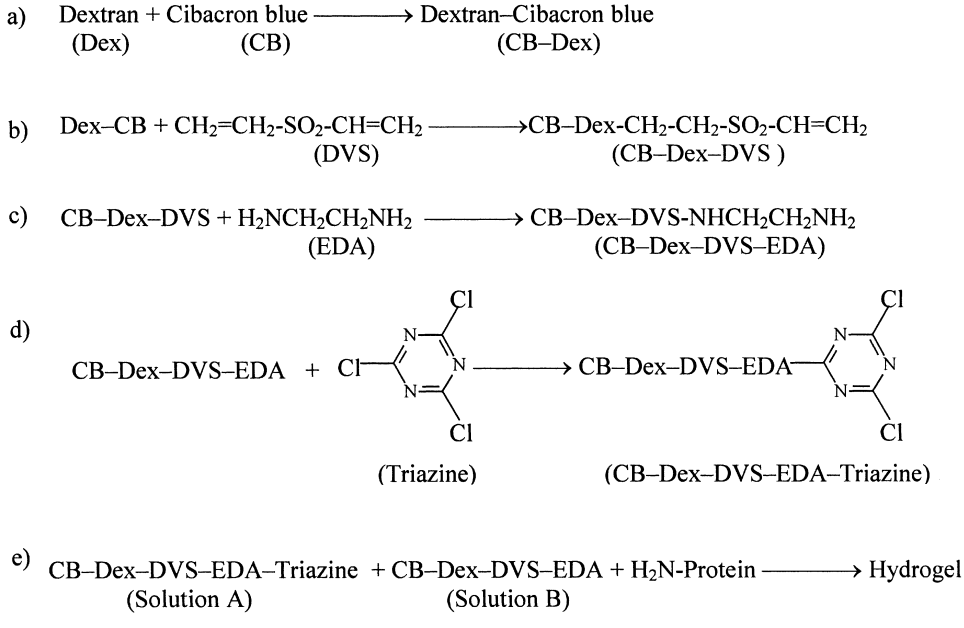

Figure 1. Synthetic scheme for Cibacron-blue–lysozyme hydrogel. Abbreviations are as defined in Materials and Methods.

and was then stopped by the addition of concentrated HCl to lower the pH to below 4 (the result will be referred to as CB–Dex–DVS).

Attachment of Triazine to CB–Dex–DVS

First, 2.5 g ethylenediamine dihydrochloride was added to the above mixture, followed by the addition of 10 M ethylenediamine (EDA) to adjust the pH to 8–9; the reaction was allowed to proceed at room temperature for the next 48 h. The solution was then dialyzed extensively and freeze-dried to concentrate to a volume of 28 ml. The ethylenediamine conjugate will be termed CB–Dex–DVS–DEA. To this solution, 0.30 g cyanuric chloride (triazine) was added and the reaction kept at room temperature for the next 24 h. The solution was centrifuged at 4,000*g* for 20 min at 4°C and the supernatant used for the preparation of the hydrogel membranes; the dextran–triazine conjugate preparation will be referred to as solution A.

A similar procedure was followed to make a CB–dextran–DVS–EDA derivative using dextran of 43 kDa; the final solution will be referred to as solution B.

Formulation of the Hydrogels

Gels were produced using the method described by Tang et al. (2003): 1.0 g of solution A was mixed with 1.0 g of solution B, followed by 0.6 ml 0.8 M $NaHCO_3$, and stirred at room temperature for 10 min. Then 80 mg lysozyme in 1.4 ml water was added and the mixture stirred for a further 15 min before vacuum degassing. This solution was poured into a mold containing a nylon gauze, pore size 0.1 mm, to add mechanical strength. The mixture was allowed to gel at 25°C for 16 h, resulting in a 0.5 mm membrane. The membrane was removed from the mold, washed with 20 mM Tris buffer, pH 7.4, containing 0.1 M NaCl and 0.01% NaN_3, and stored in this buffer at 4°C until used.

Protein Transport Experiments

The transmembrane transport of protein was studied using a diffusion cell consisting of donor and receptor chambers of equal volumes of 4.4 ml. Gel membranes with a surface area of 4.6 cm^2 were mounted between the two chambers. The proteins used for transport studies were cytochrome C and hemoglobin with molecular weights of 12 kDa and 60 kDa, respectively. Once the membranes were mounted, both chambers were filled with 20 mM pH 7.4 Tris buffer as above. The donor chamber was connected to the protein reservoir via a pump. The receptor chamber was connected to a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan), to allow protein diffusion across the membrane to be automatically monitored and logged from optical density changes. The amount of protein diffusing across to the receptor chamber was calculated using calibration data for each of the proteins at the chosen wavelength. The effect of NAD was investigated by varying NAD levels in the protein solution feed to the donor chamber and following the resultant changes in transport rate.

For diffusion experiments with cytochrome C, the protein (1.0 mg/ml) was dissolved in 20 mM pH 7.4 Tris buffer containing 0.1 M NaCl and 0.01% NaN_3. Transport was monitored at 25°C in a water bath. The cytochrome-C concentration on the receptor side was determined at 410 nm. For diffusion experiments with hemoglobin (1.0 mg/ml), absorbance was monitored at 418 nm.

RESULTS

Specificity of Membrane Response

The specificity of the response characteristics of the Cibacron blue/lysozyme hydrogel membranes was explored using NAD as a specific competitor and oxidized glutathione (GSSG) as a control. GSSG shows no affinity binding to lysozyme, but has a similar molecular weight as NAD. Figure 2 shows the diffusion curves for cytochrome C in the presence of buffer and buffer containing NAD or GSSG, through a 0.5 mm membrane.

In control experiments, protein buffer solution was used without NAD or GSSG. In initial experiments with cytochrome C there was little background diffusion in the control system. Addition of 20 mM GSSG to the donor side of the diffusion cell caused only a small increase in the rate of diffusion. However, when 20 mM NAD was used a substantial increase in the cytochrome-C diffusion rate was observed (Fig. 2). The lag time observed between addition of NAD and increased diffusion was ~20 min. This includes 4.0 min for the cytochrome C solution to be

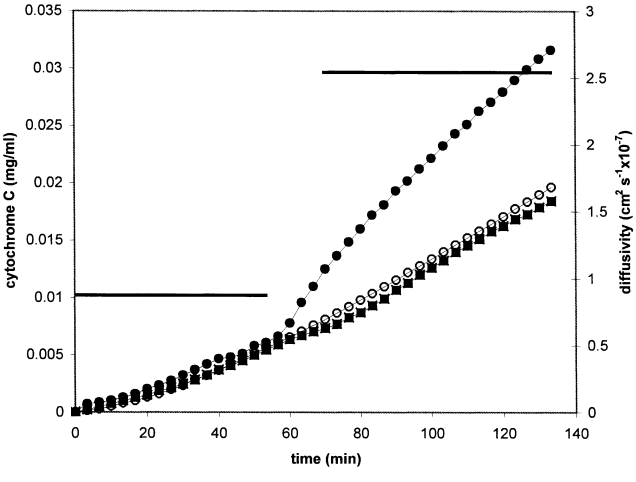

Figure 2. Diffusion curves of cytochrome C through Cibacron blue–lysozyme hydrogel. Response to 20 mM NAD and 20 mM GSSG. NAD or GSSG was added to the reservoir of the diffusion cell at 40 min. No effector ○, NAD, ●, GSSG ■, solid line shows diffusivity with and without NAD as an effector.

pumped from the reservoir into the diffusion cell followed by a mixing equilibration in the donor reservoir, during which the NAD concentration was increasing. Therefore, the response time for the membrane is substantially less than 15 min.

In order to confirm the specificity of the hydrogel membrane, a second, larger protein, hemoglobin, was used. The molecular mass of hemoglobin is about five times that of cytochrome C. Therefore, larger gel pores are required for diffusion. Figure 3 shows the release of hemoglobin in response to NAD and GSSG in the hydrogel membrane. As with cytochrome C, GSSG did not significantly influence the release of hemoglobin. However, NAD addition again resulted in a significant increase in the rate of protein diffusion across the hydrogel membrane.

These results demonstrate that the principle of a responsive membrane based on the competitive displacement of affinity crosslinks is effective, and that the membrane response is specific to the anticipated competitor (NAD). The longer response times observed with hemoglobin compared with cytochrome C are consistent with differences in their respective molecular weights.

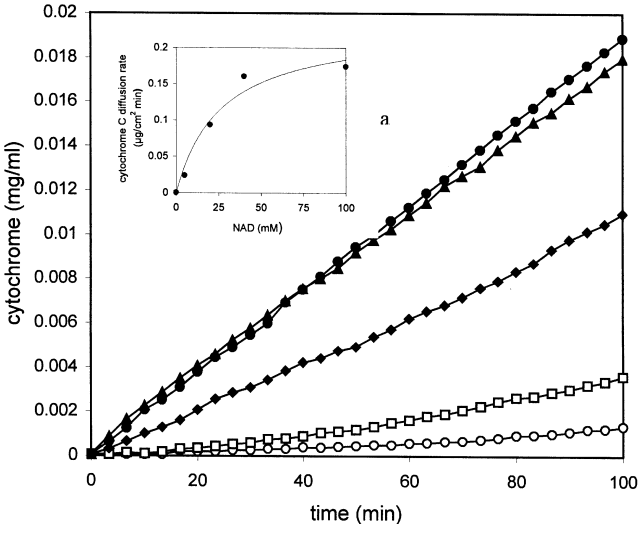

Figure 4. Diffusion curves of cytochrome C in response to NAD concentration. 0 mM (○), 5 mM (□), 20 mM (♦), 50 mM (▲), and 100 mM (●). The cytochrome C concentration was 1.0 mg/ml. Inset: Plot of Cytochrome C diffusion rates (corrected for background diffusion) against NAD concentration; the curve is a least-squares fit to the Langmuir equation (maximum rate = 0.23 μg cm^{-2} min^{-1}, half saturation constant = 26.5 mM).

Effect of NAD Concentration on Transport Rate

Cytochrome C and hemoglobin were used to examine diffusion across the hydrogel membrane in response to changes in NAD concentration. Figure 4 shows cytochrome C transport through the membrane when NAD concentrations were varied between 0–100 mM.

To avoid artifacts arising from diffusion lags after solution changes, a preincubation period was allowed before data collection took place. In the experiments without NAD, there was only a low diffusion rate through the membrane (0.012 μg $cm^{-2}min^{-1}$). However, when NAD was added to the protein reservoir there was an obvious increase in the concentration time profiles obtained up to a concentration of 100 mM (Fig. 4). The rate of diffusion in response to NAD concentration is shown in Figure 4a, indicating a half saturation constant around 20 mM.

When the larger hemoglobin was used for protein diffusion experiments, similar results were obtained (Fig. 5). As expected, the diffusion rates of the control experiment with hemoglobin and the maximum rate observed in the

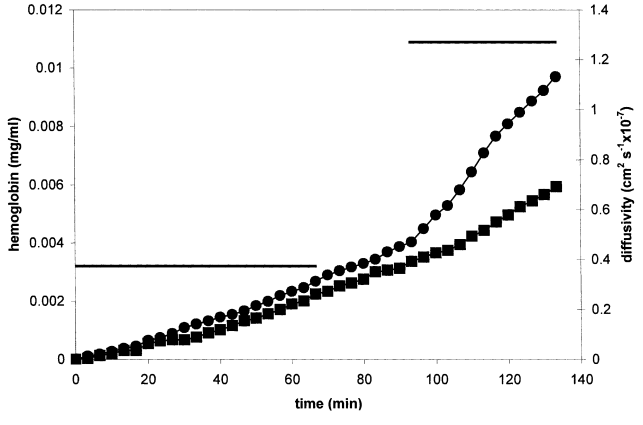

Figure 3. Diffusion curves of hemoglobin through the Cibacron blue–lysozyme hydrogel membrane. Response to 20 mM NAD and 20 mM GSSG, respectively. NAD or GSSG was added to the reservoir of the diffusion cell at 70 min. NAD, ●, GSSG ■, solid line shows diffusivity with and without NAD as an effector.

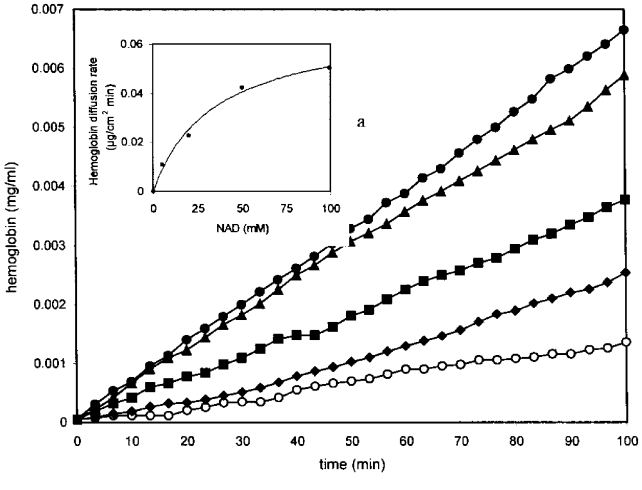

Figure 5. Diffusion curves of hemoglobin in response to NAD concentration. 0 mM (○), 5 mM (♦), 20 mM (■), 50 mM (▲), and 100 mM (●). The hemoglobin concentration was 1.0 mg/ml. Inset: Plot of hemoglobin diffusion rate (corrected for background diffusion) against NAD concentration; the curve is a least-squares fit to the Langmuir equation (maximum rate = 0.068 μg cm^{-2} min^{-1}, half saturation constant = 34.5 mM).

presence of NAD were both lower than those obtained with cytochrome C. The response of diffusion rate to change in NAD concentration also follows a saturation curve (Fig. 5a). It is noteworthy that the value of the half-saturation constant for NAD is similar to that when cytochrome C is the diffusate—this demonstrates the specificity of the membrane response to NAD.

Reversibility of the NAD Response

The reversibility of the NAD effect was studied by switching between protein solutions with and without NAD at the half saturation concentration (20 mM). In experiments with cytochrome C (Fig. 6), protein (1.0 mg/ml in the reservoir, 100 ml) was first used for the first 42 min, providing the baseline diffusion curve. At 100 min, the protein solution was replaced with one containing 20 mM NAD. The cell was flushed with 6.0 ml of the new solution before recirculation was resumed to ensure no dilution of NAD. As the total amounts of cytochrome C and NAD in the reservoir were much higher than the corresponding amounts in the receptor side of the diffusion cell, the effective driving concentration can be assumed to be constant throughout the run. A resultant increase in the rate of cytochrome C diffusion occurred about 30 min after the switch to an NAD-containing solution. This cycle was repeated: at 210 min, pure protein solution replaced that containing 20 mM NAD, and at 300 min protein solution containing 20 mM NAD was reintroduced.

At each solution change there is a clear diffusion lag before the transport rate responds to the NAD concentration, but the effect is the same for both cycles: addition of NAD increases the rate, removal reduces it.

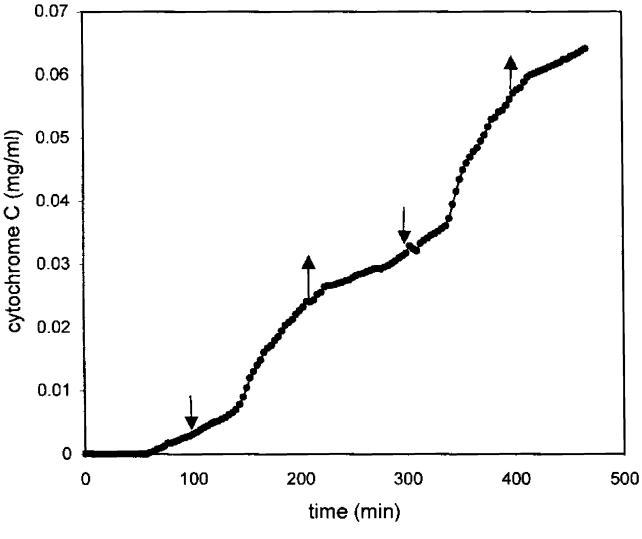

Figure 6. Reversibility of diffusion of cytochrome C through a hydrogel membrane. Diffusion curves for cytochrome C. NAD was added at 100 min and 300 min (down arrows), and removed at 210 min and 400 min (up arrows).

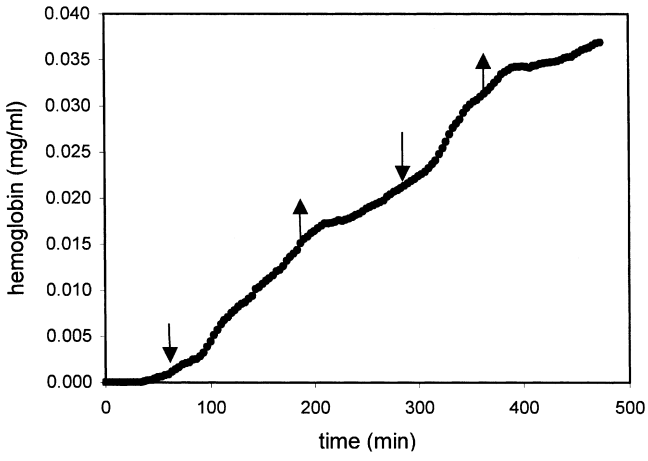

Figure 7. Reversibility of diffusion of hemoglobin through a hydrogel membrane. NAD was added at 90 min and 280 min (down arrows), and removed at 180 min and 367 min (up arrows) (temperature: 25°C).

These experiments were again repeated with hemoglobin following a similar procedure (Fig. 7). After free diffusion of hemoglobin for 60 min, a hemoglobin solution containing NAD was used to replace the pure hemoglobin solution in the donor side of the cell. After a lag of 25 min an increased rate of diffusion was reached. At 180 min, pure hemoglobin solution was used to replace the NAD-containing hemoglobin solution in the donor side. Again, after a lag period the rate of diffusion decreased as the NAD was eluted from the donor chamber. A repeat of this procedure was executed and a similar response observed. The results obtained with both proteins show that the NAD-sensitive membrane exhibits a reversible response in protein diffusion in response to changes in competitor concentration.

DISCUSSION

The goal of this study was to demonstrate the feasibility of synthesizing affinity-based responsive membranes where both ligand and receptor are attached to an inert polymer backbone.

The first stage aimed to establish 1) that permeability as evidenced by protein diffusion changed in response to concentration changes of specific affinity competitors, and 2) that this effect was specific. The results presented in Figures 2 and 3 confirm this to be the case. However, it is apparent that the porosity of the gel in the absence of competitor does not totally exclude the protein in the control experiments, suggesting that a higher degree of covalent crosslinking is desirable. There was also a slight increase in diffusion observed with the addition of the GSSG control, which may result from nonspecific interactions (e.g., charge effects).

The effect of NAD concentration on the diffusion of the different-sized proteins is shown in Figures 4 and 5. The inset figures show transport rate as a function of NAD

concentration and indicate a half-saturation constant around 20 mM. This is consistent with the displacement of a weak affinity interaction. As the size of the protein increases, the ratio of selective to intrinsic diffusion also increases. This suggests that, in addition to optimizing the base level of affinity plus covalent crosslinks to ensure zero intrinsic diffusion, porosity should also be optimized with respect to maximizing the selective transport.

The final objective was to assess the reversibility of the membrane response. The results described in Figures 6 and 7 confirm that membranes show a reversible response to stepwise changes in NAD concentration. Although a significant lag time (>20 min) was observed in these responses, at least 50% of this is attributable to system dead time and mixing effects. Ignoring extraneous effects, response times will be a function of a number of parameters. The diffusivities of effector and transported solute coupled with intrinsic porosity and membrane thickness will all influence response times. However, a potentially more significant effect results from the chain relaxation time, where the rate of change in gel properties is essentially limited by the effective diffusivity of the polymer chains within the hydrogel matrix. Hassan et al. (1997) demonstrated these effects in both glucose and pH-sensitive gels, finding that relaxation times were similar in both systems despite significant differences in effector diffusivity. In their study there was a 2–5-fold difference in the opening and closing rates of the gel. However, the results presented here for reversibility experiments in Figures 6 and 7 do not show such a marked difference in response to addition and removal of effector, possibly as a result of a higher crosslinking density. For any given application the degree of crosslinking will be constrained by the need to eliminate background diffusion, hence the sole adjustable variable will be membrane thickness. The reported thickness of 0.5 mm was constrained by mechanical properties. However, alternative support polymers with improved strength should allow this to be reduced.

CONCLUSIONS

Lysozyme/Cibacron blue-coupled hydrogel membranes were synthesized that are capable of releasing protein in a dose-dependent manner to changes in NAD concentration. Unlike gel-sol diffusion systems, where solute release occurs because of a phase transfer of a gel to a liquid (Qiu and Park, 2001; Obaidat and Park, 1997; Tanna et al., 1999), the membranes described here retain structural integrity even in the presence of elevated competitor concentrations and do not need external support membranes.

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