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Preparation of pore-filled responsive membranes using dextran precipitation

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ABSTRACT

A novel method for the preparation of a composite pore-filled membrane based on self-assembled hydrogels is described. Responsive pore-filled membranes based on mixtures of dextran (molecular weight 6000) and the glucose binding lectin Concanavalin A (ConA) were prepared. The transport properties of these membranes were characterised using a twin chamber diffusion cell with Cibacron blue as a tracer solute. As the ConA molecules introduce affinity cross-links into the gel structure by binding to terminal glucose moieties on the dextran chains the mesh size increased as the physical cross-link density of the hydrogel decreased with increasing competing glucose concentration. This increased the gel-phase diffusion coefficient for the tracer with results obtained for the effect of glucose on transport rates showing a linear increase up to a concentration of 40 mM. Further increases in D-glucose concentration lead to a subsequent decrease in transport rate which is attributed to the effects of gel swelling increasing the diffusion path length. Control experiments conducted with L-glucose, which does not bind to ConA, showed no increase in transport rate confirming a specific competition effect.

1. Introduction

Hydrogels are cross-linked networks of hydrophilic homopolymers or copolymers, containing large fractions of water in a three-dimensional structure. They are stabilised by the presence of cross-links, entanglements, or crystalline regions [1,2] and have been widely used in biomedical and pharmaceutical applications owing to their mechanical similarity to natural tissues, and their good biocompatibility [3,4].

A particular attraction for hydrogels in drug delivery applications is the ease with which they can be derivatised to introduce functional groups which allow the preparation of responsive materials. For example a range of D-glucose-sensitive systems has been developed by the authors and others with the aim of producing a zero-energy-requiring self-regulated insulin delivery system to act as an artificial pancreas, administering the necessary amount of insulin in response to changes in patient blood glucose concentration [2]. Three distinct approaches have been examined:

(1) Permeability changes arising from the catalytic action of immobilized glucose oxidase in a pH-responsive hydrogel [5].
(2) The use of insulin chemically modified to introduce glucose moieties, which bind specifically to the lectin ConA such that insulin is competitively displaced from immobilised ConA by soluble glucose [6].
(3) The use of ConA to cause affinity gelation of a polymer containing terminal or pendant glucose moieties to form a responsive hydrogel [7]. This avoids problems associated with a derivatised form of insulin.

There are two significant technical problems with using affinity gels based on protein receptors in release applications: firstly the capacity of the gel for protein drugs such as insulin limits the delivery period, and secondly the coupling chemistry needed to incorporate the biological component into the responsive gel precludes inclusion of protein-based drugs during gel synthesis. These problems can be avoided by using the gel in a membrane configuration to act as a controllable regulator for release from a separate reservoir.

This can be achieved in three ways.

1.1. Composite structures

For responsive systems where changes in transport properties result from a sol/gel transition, the active components need to be retained by an ultrafiltration membrane to prevent loss of material while the responsive phase in a sol state. For example, mixtures of ConA with glucose-containing polymer chains can be made to undergo sol–gel phase transformations depending on the glucose concentration in the environment. It has been shown that diffusion of insulin through the solution (sol) phase is faster than that through

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the hydrogel (gel) phase, and that insulin release can be controlled as a function of the glucose concentration in the environment [8]. In addition to the use of diffusive membranes to retain the polymer components, leakage can also be reduced by coupling the receptor protein to a larger polymer preparation, e.g. a carbomer [9].

1.2. Direct casting

In order to remove the need for containment membranes, we have cross-linked ConA with dextran to form a hydrogel which could be cast as a flat sheet membrane [10]. This material was mechanically weak but could be reinforced using internal support gauze. In transport studies membrane permeability shows a reversible change with the addition and removal of D-glucose—there was no evidence of ConA leakage from the gel.

1.3. Pore filled systems

To improve the mechanical properties of membranes based on soft gels a number of groups have developed techniques for casting hydrogels within the pores of preformed micro-porous membranes [11]. These composite membranes have been investigated for a range of applications. As the transport properties of these membranes will depend on the polymer phase within the pores, transport will still depend on hydrogel cross-link density and charge effects [12].

An attractive route to the facile formation of pore-filled membranes is offered by the work of Stenekes et al. [13] who reported the spontaneous formation of insoluble beaded precipitates in highly concentrated solutions of 6000 g mol^{-1} dextran. Dextran is attractive for drug delivery systems in that it is widely used for other medical purposes. The ability for low molecular weight solutions to aggregate into macroscopic structures without the need for additional reagents increases their attractiveness for drug delivery applications.

In a recent study we have investigated the kinetics of formation and stability of these aggregates [14] and have found that, while not dissolving at physiological temperatures, the beads (diameter $\cong$6 μm) will anneal if stored as a packed bed. The aim of the study reported here is to investigate the formation of pore-filled membranes by impregnating the pores of commercial micro-filtration membranes with concentrated solutions of 6000 kDa dextran such that beads form and then anneal within the pores to form a coherent gel layer. By including the lectin ConA in the dextran solution it was hoped that the gels formed might show glucose-responsive permeability changes similar to those previously reported for cross-linked dextran/ConA conjugates [10].

2. Materials and methods

2.1. Materials

Dextran from *Leuconostoc* ssp. was obtained from Fluka Biochemika. Concanavalin A, Trizma base, sodium azide, Cibacron blue, D(+)-glucose, and manganese chloride were obtained from Sigma–Aldrich, UK. Calcium chloride dihydrate, sodium chloride (for analysis) and magnesium chloride were obtained from Fisher Chemicals. Millipore Glass Fibre Prefilters (AP2502500, porosity 90%, diameter 25 mm) were obtained from the Millipore Corporation.

2.2. Methods

2.2.1. Pore filled membrane based on ConA-dextran hydrogel

To limit premature gel formation resulting from ConA binding causing cross-linking, ConA was dissolved in Tris buffer (50 mM, pH 7.4) containing D-glucose solution to saturate binding sites before the addition of dextran. 0.04 g ConA and 0.054 g D-glucose were added into 1.5 g Tris buffer solution. When the ConA was completely dissolved, 1.5 g of dextran (molecular weight 6000) was added. The mixture was stirred gently and a vacuum applied to remove entrained air bubbles. The solution was placed on the surface of the support membrane mounted in a vacuum funnel and vacuum was again applied. When the glass–fibre membrane was saturated with dextran solution, it was removed from the sintered glass filter and soaked in excess dextran solution in a small Petri dish. After 2 days incubation at 20 °C, the dextran solution became turbid as beads formed. Image analysis of light micrographs obtained using a Nikon Diaphot 300 microscope showed these to have a radius 2.9 ± 0.8 μm and to be approximately spherical in shape.

Once formed, external beads could be washed from the surface of the membrane leaving the entrapped beads to anneal into a coherent gel layer. Membranes were then stored at 4 °C for a further 3 days to allow annealing to occur. The resultant pore-filled membranes were soaked in a vast volumetric excess of buffer for 12 h to remove glucose prior to use. Images of membranes taken before and after gel impregnation were again taken using a Nikon Diaphot 300 microscope. Based on geometric and gravimetric measurements we estimate a membrane gel loading of 150 ± 15 mg cm^{-2}.

2.2.2. Trace response experiments

Membranes were mounted into a diffusion cell comprising of a donor and a receiver chamber [10]. The complete system comprised the diffusion cell, a UV detector, recirculation pumps, feed reservoir and a thermostatic water bath allowing donor and receiving solutions to be recycled from a holding reservoir across each face of the membrane. The UV detector was used to monitor changes in tracer concentration in the receiving solution arising from diffusive transport of tracer across the membrane. Cibracron blue was used as a tracer in membrane transport experiments as a low molecular weigh species that provides a more sensitive test of membrane permeability than larger protein tracers that were used for initial trials. To prevent covalent coupling of the dye to the dextran, it was inactivated prior to use by incubating in 0.1 M sodium hydroxide for 3 h prior to adjusting pH to that used in transport experiments (7.4) using hydrochloric acid.

To allow thermal equilibration Tris buffer (50 mM, pH 7.4) solutions with and without deactivated Cibacron blue were placed in the thermostatic bath at 37 °C for 2 h prior to use. A further 30 min was allowed for the baseline to stabilize after recycle flow was started (1.3 g/min) before transport experiments were conducted. Buffer containing deactivated Cibacron blue was introduced into the reservoir to give a tracer concentration of 0.1 mM. A further 90 min was allowed to elapse before D-glucose was added at the required test concentration.

2.2.3. Protein leakage

As the conA used to generate affinity cross-links was not covalently coupled to the dextran that forms the gel matrix the possibility of conA leakage could not be ignored. To investigate this, the system was run with just buffer in both donor and receiver chambers for 2 weeks while the optical density of the solution on the receiver side was monitored by following changes in optical density at 280 nm.

3. Results

3.1. Membrane characterisation

Microscope images of bead formulations were taken for precipitates formed in the absence of membranes. Fig. 1 shows the

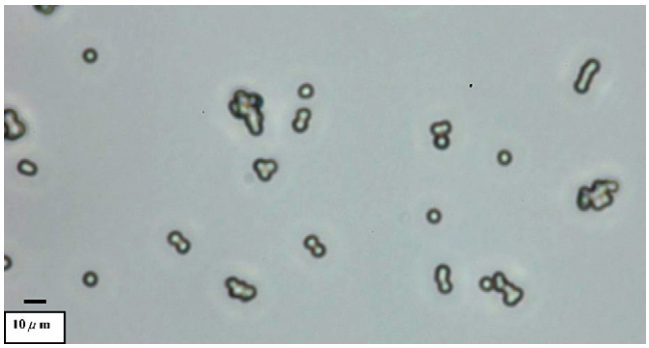

Fig. 1. Image of beads following suspension and settling in excess buffer showing beads aggregating and annealing.

initial stages of bead aggregation and annealing for dextran solutions prepared without membranes. Fig. 2a shows a comparison of the native glass–fibre membrane (in b) the composite membrane structure after annealing.

As a control, initial trace response experiments were conducted with native support membranes. Fig. 3 shows the traces obtained for three flow rates. In Fig. 4 the results of a similar experiment conducted with a pore-filled membrane are shown, indicating an approximately fivefold increase in the time for the tracer concentrations to reach equilibrium.

3.2. Glucose response

Tracer studies were conducted to examine the effect of D-glucose concentration on diffusion rates. Fig. 5 shows the absorbance variations in response to different concentration of D-glucose from 10 to 40 mM. D-Glucose solution in buffer was added to the reservoir at 150 min.

Glucose response experiments were carried out in order of increasing concentration from 10 to 100 mM D-glucose. Membranes were not changed during these experiments and were not removed from the diffusion cells between experiments. Between each glucose concentration, the solutions in both donor reservoir and receiver reservoir were drained and replaced with Tris buffer which was recirculated for 12 h prior to the next run to remove residual tracer and glucose from the membrane.

Response traces obtained for 10–40 mM glucose are shown in Fig. 5 with a clear increase in transport rate shown in each case. As shown in Fig. 6, transport rate increases with glucose concentration up to a value of 40 mM and then decreases with further increases up to 100 mM. A similar effect was observed by Tang et al. [10] for the transport of lysozyme through a glucose responsive membrane

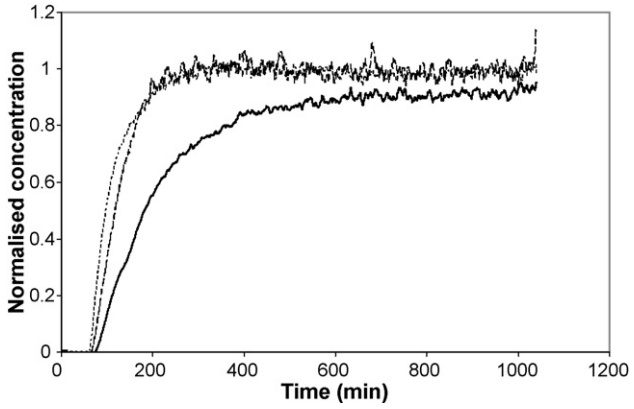

Fig. 3. Effect of recirculation flow rate on time taken for tracer concentrations to reach equilibrium across an unmodified membrane (short dash 1.24 g/min, long dash 1.02 g/min, solid 0.83 g/min).

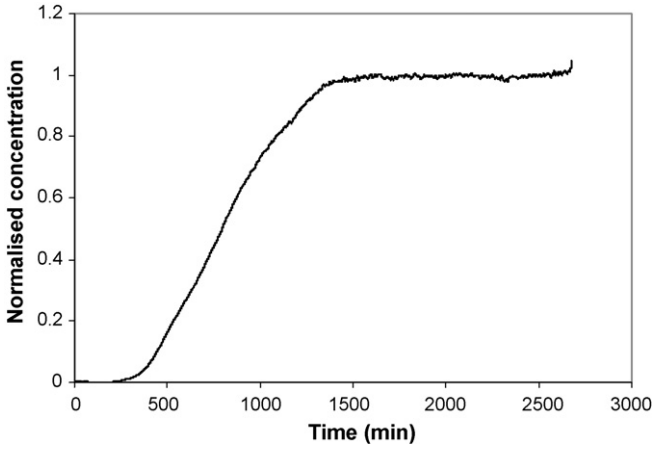

Fig. 4. Time taken for tracer concentration to reach equilibrium across a pore-filled membrane (recirculation flow 1.02 g/min).

This can be explained in terms of the displacement of affinity cross-links leading to two competing effects (i) an increase in the mesh size within the gel leading to an increase in gel phase diffusivity and (ii) an increase in gel swelling leading to an increase in membrane thickness.

As a final check the initial 10 mM trial was repeated after the 100 mM run to ensure the washing protocol used between runs was adequate. Fig. 7 shows a comparison of the first and last 10 mM runs indicating that washing is effective and that the membrane

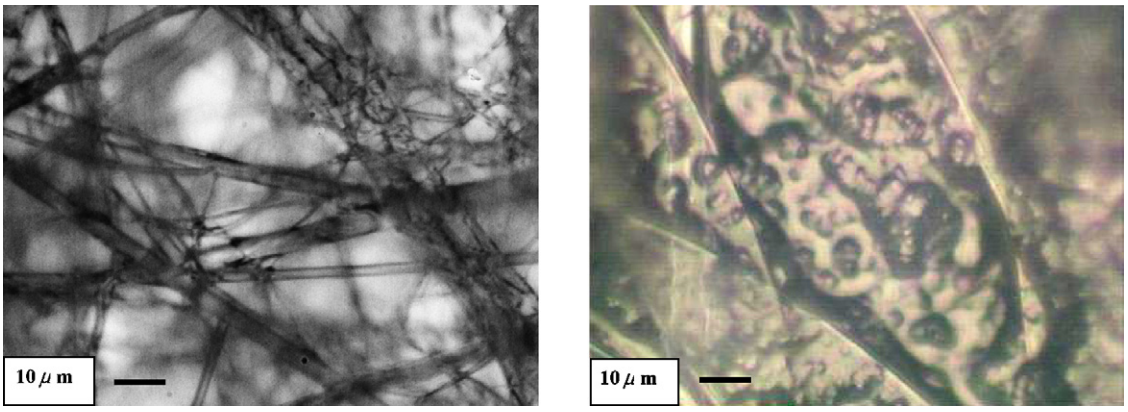

Fig. 2. (a) Micrograph of a native glass fibre membrane. (b) Micrograph of a composite membrane showing bead coalescence.

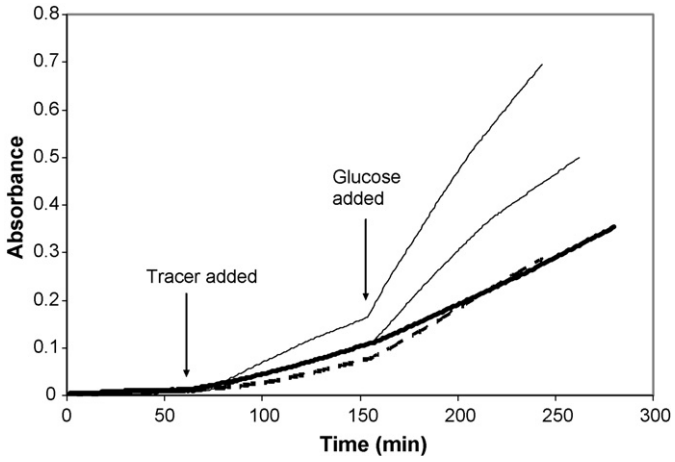

Fig. 5. Effect of D-glucose addition to the diffusion time course of tracer through a ConA dextran pore filled membrane (bold solid line 10 mM, bold broken line 20 mM, solid line 30 mM, broken line 40 mM).

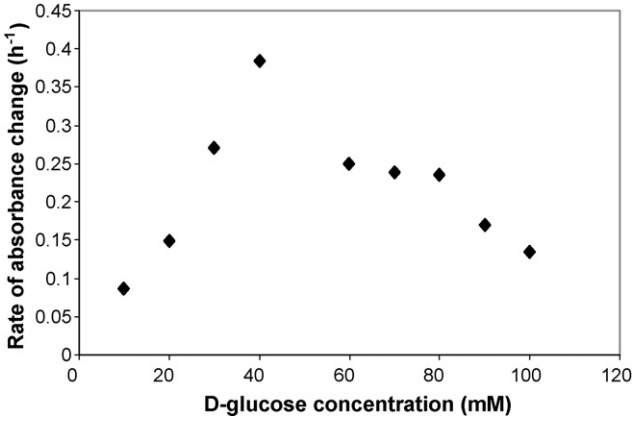

Fig. 6. Effect of D-glucose concentration on the diffusion rate of tracer through a ConA dextran pore-filled membrane.

has retained most of its functionality over a ten-day period. This was in agreement with protein leakage measurements which showed minimal evidence of protein leakage from the gel over this period.

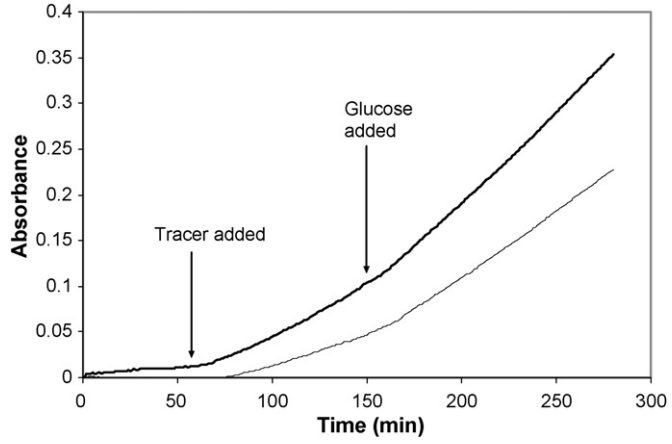

Fig. 7. Comparison of glucose effect between first (solid line) and last (broken line) experiments in the sequence.

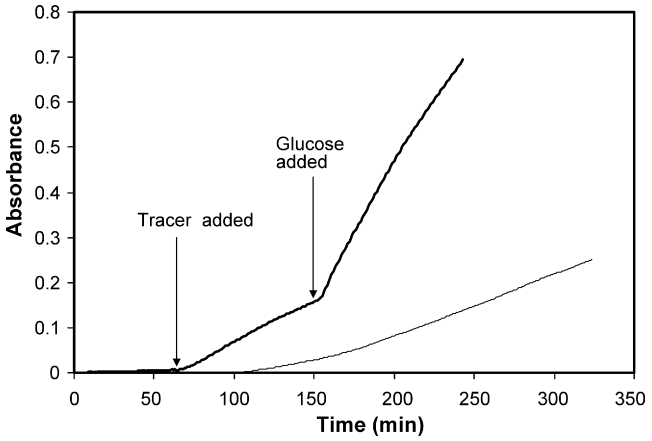

Fig. 8. Comparison of the effect of 40 mM D-glucose (solid line) and L-glucose (broken line) on membrane transport.

3.3. Specificity

The specificity of the dextran–ConA hydrogel was determined by comparing the responses obtained using D-glucose and L-glucose, respectively. D-Glucose is known to bind to ConA and hence would be expected to reduce the cross-link density of the dextran beads. This increases the mesh size and hence facilitates the diffusion of the Cibacron blue tracer. In contrast, L-glucose does not interact with ConA and, if the response is specific, should not result in a similar increase. As can be seen in Fig. 8, while D-glucose (40 mM) leads to a marked response as seen in Fig. 5, L-glucose at the same concentration has a negligible effect.

4. Conclusions

This study demonstrates a facile method for the preparation of responsive pore-filled membranes. These structures combine the mechanical strength of the support matrix with the selectivity of the responsive hydrogel. Used in combination with drug reservoirs they offer significant advantages over drug release membranes based on hydrogels alone. If constructed with microporous membranes formed from biocompatible polymers the hydrogels based solely on dextran should result in minimal toxicity problems. While the ConA used in this evaluation is cytotoxic and immunogenic a similar response might be expected from a human sourced alternative, e.g. mannose-binding lectin (MBL) which forms part of the innate immune system and shows binding specificity towards a number of sugars including D-glucose. As MBL deficiency has been associated with a number of disease states, therapies based on MBL replacement have already been investigated in a number of clinical trials [15] paving the way for its evaluation in insulin release applications.

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