Available online at www.sciencedirect.com
SCIENCE DIRECT
Reactive & Functional Polymers 66 (2006) 757–767
REACTIVE & FUNCTIONAL POLYMERS
www.elsevier.com/locate/react

Synthesis and characterization of a D-glucose sensitive hydrogel based on CM-dextran and concanavalin A

R. Zhang [a], M. Tang [a], A. Bowyer [b], R. Eisenthal [c], J. Hubble [a,*]

[a] *Department of Chemical Engineering, University of Bath, Claverton Down, Bath BA2 7AY, UK*
[b] *Department of Mechanical Engineering, University of Bath, UK*
[c] *Department of Biology and Biochemistry, University of Bath, UK*

Received 20 December 2004; received in revised form 4 August 2005; accepted 17 November 2005
Available online 11 January 2006

Abstract

A facile method is described for the preparation of D-glucose-sensitive hydrogel membranes based on cross-linking carboxymethyl dextran (CM-dextran) with the glucose binding lectin concanavalin A (ConA) using carbodiimide chemistry. The mesh size of these hydrogels is determined by both covalent cross-links and affinity interactions between ConA and terminal glucose moieties on the dextran chains. Competitive displacement of the affinity interactions by D-glucose leads to changes in both morphology and permeability. The carbodiimide coupling chemistry has the advantage that it introduces no potentially cytotoxic groups into the gels formed rendering them more suitable for potential in vivo applications.

Infrared spectra of the hydrogels suggest that, in addition to direct cross-linking of CM-dextran chains, gels are also stabilized by formation of amide bonds between amine groups in the ConA and carboxyl groups in CM-dextran. This suggests that the mechanical properties of the gels can be modified by the inclusion of inert proteins to increase the density of covalent cross-links.

The permeability of the hydrogels, as shown by protein diffusion, increases in response to changes in the D-glucose concentration of the external medium, causing competitive displacement of the affinity cross-links. Sequential addition and removal of external glucose in a stepwise manner showed that these permeability changes are reversible. Results obtained using isothermal titration calorimetry confirmed the competitive binding between ConA-dextran and D-glucose this was confirmed to be a biospecific effect by the observation that L-glucose was not bound by free or dextran coupled ConA. Scanning electron microscopy of samples prepared using cryofixation and cryofracturing techniques showed that observed changes in permeability correlate well with glucose dependent structural changes in the gel.

The effects of varying pH and ionic strength show that ion exchange effects also influence diffusion rates. However, an advantage of the synthetic route described is that the intrinsic charge density of the hydrogels produced can be tailored to the specific interaction by varying the degree of carboxylic substitution on the original dextran. The ease of preparation and control of final gel properties allowed by this approach offers possibilities for a range of biotechnological applications including drug delivery and biosensors.

Keywords: Responsive; Lectin; Insulin; Control

* Corresponding author. Tel.: +44 1225 386221; fax: +44 1225 386894.
E-mail address: j.hubble@bath.ac.uk (J. Hubble).

1381-5148/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.reactfunctpolym.2005.11.003

1. Introduction

An ideal drug delivery system should be capable of adjusting delivery rate to match a time varying level of demand. To meet this ideal, closed-loop delivery systems have been developed that are self-regulating, i.e. they respond to changes in the local environment, such as the presence or absence of a specific molecule [1]. A prominent application of this approach is development of systems that can autonomously release insulin in response to changes in monitored blood glucose levels. An attractive approach for controlling the delivery of insulin is based on the use of competitive binding to change the permeability of a release matrix [2], e.g. mixtures of the plant lectin concanavalin A with specific polysaccharides can biospecifically interact in a reversible gelation process that can be used to control insulin release [3–5].

There have been two separate approaches that utilize this effect. The first involves the preparation of glycosylated insulin, which can bind to concanavalin and be competitively displaced by free glucose [6]. The displacement of glycosylated insulin from ConA was found to be proportional to the external glucose concentration. A similar approach was development by Kim and coworkers, using a polymeric membrane permeable to glucose and glycosylated insulin and non-permeable to ConA [7,8]. Control of the binding constants of glycosylated insulin derivatives to ConA allows control of the response to external glucose levels. While this approach is highly elegant, the requirement for chemically modified insulin increases cost and could potentially modify the therapeutic response.

The alternative approach uses glucose-containing polymer and glucose receptor molecules to prepare glucose-sensitive hydrogel systems. Taylor [9] proposed a system where the ConA is mixed with polysaccharides such as polysucrose [10,11], dextran [12–14], and glycogen [15,16]. Biospecific binding between ConA and polysaccharide lead to the formation of a gel which changes its viscosity in response to free glucose concentration, thus providing the switch for controlling insulin diffusion rate. The release mechanism is repeatable, releasing insulin in response to several free glucose fluctuations, in a manner that mimics the in vivo feedback mechanism of pancreatic cells. A problem encountered in these studies was the significant leakage of ConA during the low viscosity phases. In a more recent refinement to reduce leakage, ConA was first covalently bonded carboxylic moieties on Carbopol 934P NF and 941P NF using carbodiimide chemistry before mixing with dextran, to produce a glucose-sensitive formulation that still shows a transformation from gel to sol in the presence of free glucose [17,18].

Park and coworkers [19–21] used a similar approach to show that polymer-bound glucose and ConA could form a gel capable of reversible sol–gel transition for the regulation of insulin release. In this case, mixing glucose-containing polymers with ConA formed the gel. Glucose containing polymer chains were synthesized by co-polymerizing allyl glucose and acrylamide or vinylpyrrolidone. The glucose-sensitive hydrogel layer was sandwiched between porous poly[hydroxyethyl methacrylate] membranes. Although this system controls the release of insulin in response to glucose concentration, the response time may be too slow for therapeutic applications.

We previously reported the synthesis of a mechanically stable, glucose responsive hydrogel membrane, based on copolymerising ConA with two dextrans of different molecular weights [22]. The larger dextran is used to provide structural support, while the second smaller one, to which the ConA is coupled, is provides the necessary internal flexibility. This is conceptually similar to the work of Miyata et al. [23], who describe an antibody/antigen-based gel that swells in response to free antigen. To prepare this hydrogel, gels were formed via a nucleophilic replacement reaction in a triazine ring, such that both gel formation and ConA fixation occurred at the same synthesis step. This hydrogel demonstrated mechanical stability, changes in permeability in response to changes in glucose concentration, and showed negligible ConA leakage over extended periods. However, its major drawback for use in biomedical application is the presence of the cytotoxic triazine moiety.

To address the problem of potential cytotoxicity, we have applied a synthetic approach following one recently reported by us for the fabrication of a pH-responsive dextran hydrogel. This is based on the intermolecular cross-linking of carboxymethyl dextran (CM-dextran) using 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) [24]. EDC was used as it is not incorporated into the cross-linked structure, but is simply changed to a water-soluble urea derivative as the reaction proceeds. The cytotoxity of the urea derivative has been found to be quite low compared with that of EDC [25] as shown by

the work of Tomihata and Ikada [26] who synthesized an uncharged hydrogel for tissue engineering applications using EDC cross-linking of hyaluronic acid. We also demonstrated that the CM-dextran hydrogel produced by this method could be used as a support for the attachment and maintenance of primary human dermal fibroblast cultures. Cell cultures over a period of 7 days showed normal morphological and proliferative capabilities [unpublished observations].

Our primary targets here are to demonstrate that a novel D-glucose sensitive hydrogel membrane can be produced using this approach, i.e. by incorporating ConA into a CM-dextran hydrogel network using carbodiimide chemistry such that the protein, distributed throughout the hydrogel, can reversibly modify permeability of macromolecules in response to changes in environmental D-glucose.

2. Materials and methods

2.1. Materials

Dextran and lysozyme were obtained from Sigma-Aldrich, UK. All other chemicals were of reagent grade and obtained from Lancaster Synthesis Ltd., UK. Double distilled water was used throughout.

2.2. Hydrogel preparation

CM-dextran was prepared as described by Zhang et al. [24]. Five grams 480 kD dextran was dissolved in 75 ml distilled water and 5 g sodium chloroacetate added. The carboxymethylation reaction was initiated by the addition of 25 ml of 8 M NaOH. The reaction mixture was then diluted to 100 ml total volume with water. Carboxymethylation was allowed to proceed for 15 min at 50 °C with stirring. The reaction was then terminated by lowering the solution pH to 7 with 6 M HCl.

The product was precipitated with 300 ml absolute ethanol and allowed to stand overnight. The precipitate was dissolved in water and then exhaustively dialysed for two days against water. The dialysed solution was rapidly frozen using liquid nitrogen and lyophilized, giving a white powder.

The degree of COOH substitution in the CM-dextrans was determined by titration. The final ratio of COOH groups per dextran was 1-COOH:65 glucose residues.

To produce a ConA hydrogel, 1 g CM-dextran was dissolved in 4.2 ml water with stirring and the resulting solution degassed. EDC (210 mg) and NHS (30 mg) dissolved in 1.5 ml water was added to the CM-dextran solution and stirred for 10 min. 3.0 ml of a solution containing 130 mg ConA, 0.5 mM $CaCl_2$, 0.5 mM $MnCl_2$ and 0.5 mM $MgCl_2$ was then added. The resulting solution starts to gel after 30 min at which point it can be cast as required (in this case onto a nylon gauze support, pore size 0.1 mm, thickness of 0.5 mm mounted between two glass plates using spacers to give the required total membrane thickness).

2.3. Physicochemical properties of the hydrogel

Infrared spectroscopy of samples taken at different stages of the reaction sequence was performed using a Bruker-equinox 55 FT-IR spectrometer (Bruker, Germany). Freeze-dried samples were mixed with potassium bromide powder and pressed into tablets under vacuum. For each sample 100 scans were recorded from 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1}.

2.4. Hydrogel morphology

The morphology of the hydrogel under different conditions was examined using a Jeol 6310 (Jeol, Japan), scanning electron microscope equipped with a cryo-stage and energy-dispersive X-ray. Samples of hydrogel were clamped between two pieces of metal sheet, rapidly frozen in liquid nitrogen and then introduced into the SEM chamber pre-cooled to a temperature of −160 °C. The stage was then heated to a temperature of −80 °C to sublime off the surface water. After cooling to −160 °C, the sample was gold sputtered for 3 min. Samples were scanned at a magnification of 10,000×.

2.5. Characterization of binding interactions

The binding properties of ConA towards glucose, dextran and derivatized dextran were studied using Isothermal Titration Calorimetry (ITC) in an OMEGA ITC (Microcal Ltd., USA). The instrument was calibrated and experiments conducted in accordance with the manufacturers operating instructions.

Before use, ConA-dextran was dissolved in, and dialysed against 20 mM Tris buffer pH 7.4 containing, 0.5 mM $CaCl_2$, 0.5 mM $MnCl_2$, 0.5 mM $MgCl_2$,

150 mM NaCl, and 0.02% NaN_3 at 4 °C, filtered through a 0.22 μm PVD/PE syringe filter and the concentration (M_w 27 kD) adjusted to 0.092 mM with respect to ConA monomer. Titrant sugar samples of D-glucose, dextran (M_w 15 kD), and L-glucose were diluted to a final concentration of 27.8 mM (dextran concentration expressed in terms of glucose monomer). Titrations were run at 20 °C and all the samples degassed at 15 °C prior to use. In each case 54 injections of 5 μl of sugar solution were automatically added at 2 min intervals into the lectin solution stirred at 300 rpm. The heat evolved after each injection is measured by the cell's feedback network as differential heat effects between sample and reference cell.

Binding interactions between ConA conjugated dextran and competing D-glucose were investigated using a low molecular weight dextran backbone (12 kD) to minimize gel formation. In other respects the synthesis was as described above for the preparation of hydrogels. Conjugates were exhaustively dialysed against 20 mM Tris buffer pH 7.4 at 4 °C and filtered prior to use. The conjugation concentration of ConA-CM-dextran was determined using optical density measurements against a ConA standard curve.

In addition to direct binding measurements, the competitive displacement of bound dextran using monosaccharides was also studied. ConA, or ConA conjugated with CM-dextran was mixed with dextran (M_w 15 kD) and then titrated with D-glucose (27.8 mM). The concentrations of ConA, ConA conjugated with CM-dextran, and dextran were 0.092, 0.146, 0.139 mM, respectively, in final samples used in ITC experiments.

In all cases control experiments were carried out using identical sugar injections into a cell-containing buffer without protein and relative heats of dilution and mixing subtracted from the heats of binding relative to the test titration experiment.

Final experiment results, represented by heat developed versus cell ligand/lectin ratio, were fitted to a single binding site per monomer model using Origin ITC Data Analysis Software (Microcal Ltd.).

2.6. Protein diffusion experiments

Trans-membrane protein transport was investigated using lysozyme as the test molecule. Experiments were conducted in a diffusion cell consisting of donor and receptor chambers of equal volumes of 4.4 ml [22]. Hydrogel membranes with a surface area of 4.6 cm^2 were mounted between the two chambers. Once the membranes were mounted, both chambers were filled with 20 mM Tris buffer. The donor chamber was connected to the lysozyme reservoir via a pump. The receptor chamber was connected to a UV–vis spectrophotometer (Shimadzu 1601, Japan), to allow lysozyme diffusion across the membrane to be automatically monitored and logged from optical density changes. The amount of lysozyme diffusing across to the receptor chamber was calculated using calibration data for lysozyme at 280 nm. The effect of pH and ionic strength was investigated by varying pH of phosphate buffer and ionic strength by varying the NaCl concentration (compensating for buffer composition) in the lysozyme solution feed to the donor chamber and following the resultant changes in transport rate. All experiments were conducted at 20 °C.

3. Results

3.1. Hydrogel characterization

The hydrogel synthesis utilizes carbodiimide chemistry and is based on the formation of amide bonds between carboxylic acid groups on carboxymethyl dextran and amines, by activating the carboxyl to form an O-urea derivative. This derivative reacts readily with nucleophiles (Fig. 1). The reagent can be used to make ether links from alcohol groups, ester links from acid and alcohols or phenols, and peptide bonds from acids and amines [27]. The water-soluble derivative EDC can then be used to synthesize peptide bonds using NHS [28]. The reaction includes formation of the intermediate active ester (the product of condensation of the carboxylic group and NHS) that further reacts with the amine function to yield an amide bond.

According to the expected cross-linking mechanism, when ConA is included the activated ester bond will react with an amino acid group on the protein. In the case of D-glucose sensitive hydrogels, ConA was covalently bound to CM-dextran. A control hydrogel was made using a similar concentration of ovalbumin as the coupled protein to approximate the same covalent cross-link density without introducing the potential for affinity cross-links.

The successful incorporation of the COOH group into dextran is demonstrated by the presence of a carboxylic FT-IR band from the carbonyl (C=O) stretching of CM-dextran at ca. 1734 and

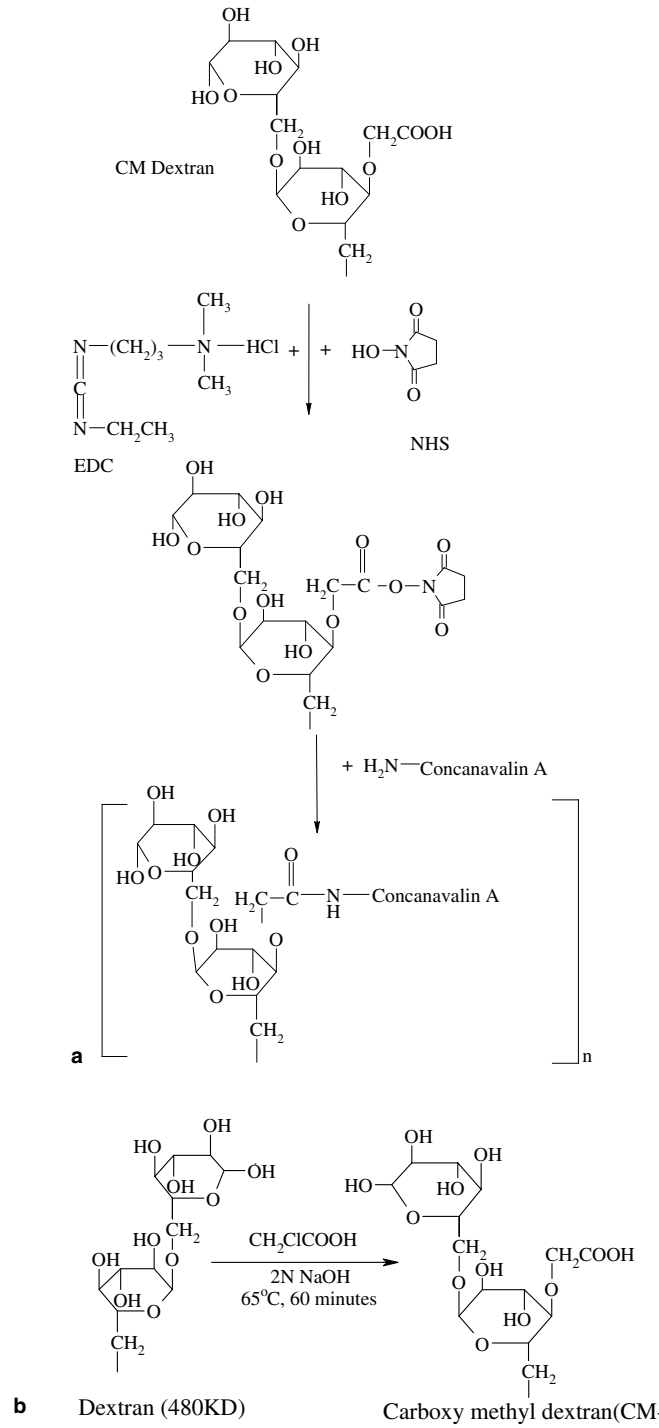

Fig. 1. The chemistry of concanavalin A hydrogel preparation based on carbodiimide coupling (a) of custom synthesized carboxymethyl dextran CM-dextran (b).

1580 cm^{-1} as shown in Fig. 2. Carboxyl groups activated by EDC/NHS can react with hydroxyl groups in CM-dextran to form ester linkages. Furthermore, in the solid phase, primary amides in ConA-hydrogel have a weak-to-medium intensity band at 1650–1620 cm^{-1} which is generally too close to the strong

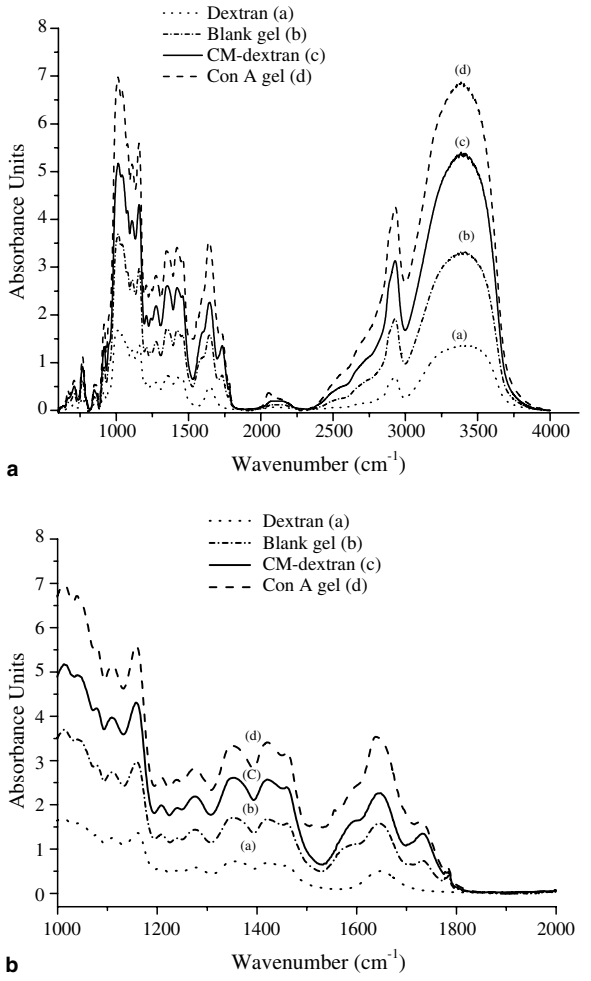

Fig. 2. Dextran hydrogels and hydrogels grafted with ConA characterized by Fourier transform infrared spectroscopy (FT-IR). The peaks from the carbonyl (C=O) stretching for both CM-dextran and gel are seen at 1734 and 1580 cm^{-1}. The peak for the carbonyl group of the gel ester bond is seen at 1775 cm^{-1}. Peak between 1570 and 1515 cm^{-1} in the ConA gel show characteristic absorption of the secondary amides in this hydrogel.

carbonyl band to be resolved. The amide II band from protein bound to dextran as seen at 1570–1515 cm^{-1} is due to a motion combining both the N–H bending and the C–N stretching vibrations of the group –CO–NH– in its *trans*-form [29].

3.2. Morphology of hydrogels

Four samples of ConA gel were prepared for SEM analysis. (a) Incubated in buffer, (b) incubated in buffer + D-glucose, (c) incubated in buffer + L-glucose, (d) incubated in buffer + D-glucose followed by washing in glucose-free buffer for 2 days. As a control a gel (e) was made with an inert protein (ovalbumin).

Results presented in Fig. 3 show that the ConA (Fig. 3a) and ovalbumin (Fig. 3e) gels have a similar morphology when incubated in buffer. Addition of D-glucose to the ConA gel leads to significant swelling (Fig. 3b), whereas no swelling was observed with the ovalbumin gel (result not shown). The response of the ConA hydrogel to D-glucose is proposed to result from changes in the pore walls such that a relatively compact structure in buffer expands resulting in a larger internal pore volume. The control test with L-glucose at a similar concentration (c) shows little evidence of gel expansion confirming that the effect is the result of a specific displacement rather than an osmotic pressure effect. Examination of a ConA gel incubated with D-glucose and then extensively washed (d) shows clear evidence of reversibility with the internal expanded pore structure returning to that seen for the control gel.

3.3. Binding capacity comparison of different sugars with concanavalin A

Isothermal titration microcalorimetry (ITC) has found extensive utility in ligand binding studies. Ligand is titrated into a solution containing the binding protein (here ConA), and the heat evolved or absorbed during binding is measured. Deconvolution of data obtained from a sequence of additions yields the binding constant, enthalpy of binding, and the stoichiometry of the interaction. Fig. 4-(panel 1) shows the raw calorimetric data for titration of free ConA with D-glucose, denoting heat evolved (negative exothermic peaks). Panel 2 shows the plot of heat evolved per injection as a function of the molar ratio of ligand to the ConA. The smooth solid line represents the best fit of the experimental data using a single binding site model. The binding constants of different ligands to ConA and CM-dextran-ConA conjugates are shown in Table 1. Comparison of the binding constants of D-glucose with free ConA and with ConA conjugated to CM-dextran shows an increase in the dissociation constant (K_D) from 6.0 to 10.6 mM indicating a reduced affinity of the conjugated ConA for free glucose. A similar result is seen for the interaction of dextran with free ConA ($K_D = 28.6$ mM) and with ConA conjugated to CM-dextran ($K_D = 81.7$ mM). When D-glucose is used to competitively displace pre-bound dextran K_D increases from 17.7 mM ConA conjugated dextran to 45.2 mM for the displacement

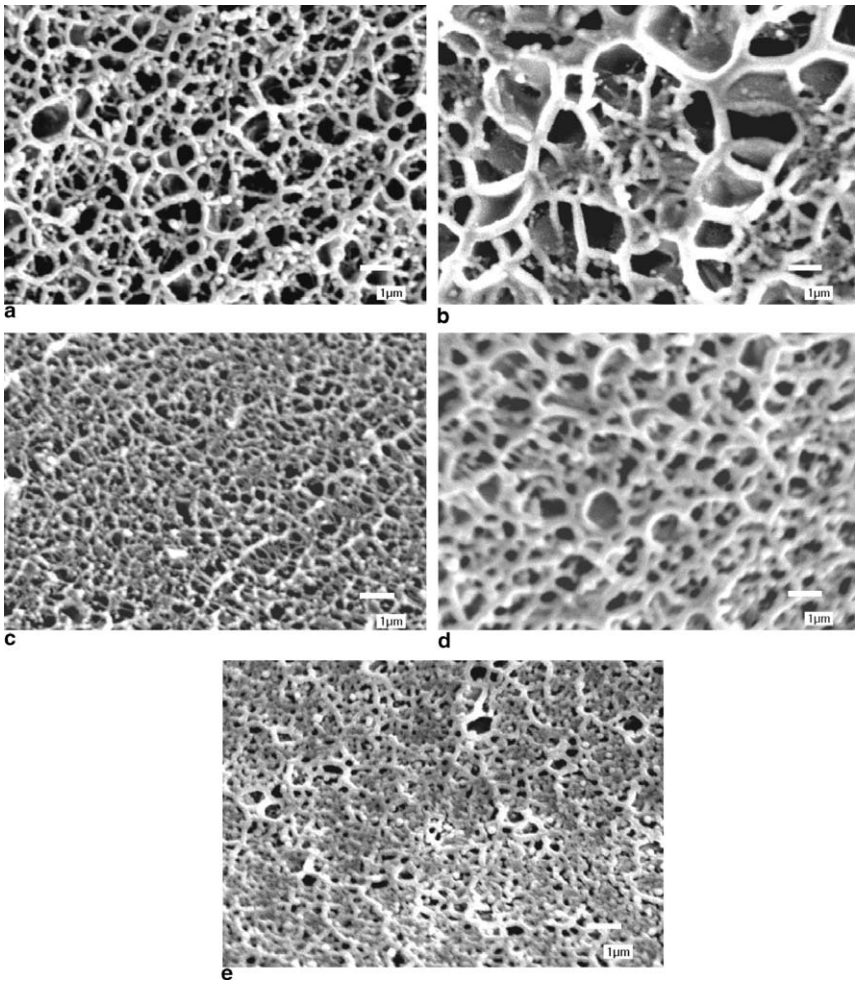

Fig. 3. Electron micrographs of hydrogels at 10,000× magnification: (a) ConA hydrogel in 20 mM Tris buffer. (b) ConA hydrogel in 20 mM Tris buffer + 0.1 M D-glucose. (c) ConA hydrogel in 20 mM Tris buffer + 0.1 M L-glucose. (d) ConA hydrogel extensively washed in 20 mM Tris buffer after exposure to 20 mM Tris buffer + 0.1 M L-glucose. (e) Ovalbumin hydrogel in 20 mM Tris buffer.

of dextran from native ConA. This is consistent with the lower association constant values obtained in direct binding titrations. Titrations with L-glucose show no evidence of binding to free ConA. However, weak binding is observed with CM-dextran-ConA suggesting that the immobilization has reduced the binding specificity of the ConA.

3.4. Diffusion characteristics of lysozyme through hydrogel

The proposed response mechanism of the D-glucose sensitive hydrogel membrane is based the competitive displacement of biospecific interactions between ConA and the glucose backbone of dextran leading to an increase in permeability. Results from both SEM and ITC experiments show that D-glucose is an effective competitor of these internal affinity links, and that displacement of ConA dextran interactions within the gel leads to clearly observable changes in morphology.

For an effective delivery system it is necessary to show that these morphological changes lead to changes in gel permeability and that these changes are both specific and reversible. Fig. 5 compares the permeability of membranes produced using both ConA and ovalbumin as an inert control. Both gels show negligible transport of the protein lysozyme (molecular weight 17 kDa) for the first 100 min. After the addition of 0.1 M glucose as a competitive displacer the difference is marked, with the ConA gel showing a clearly enhanced permeability.

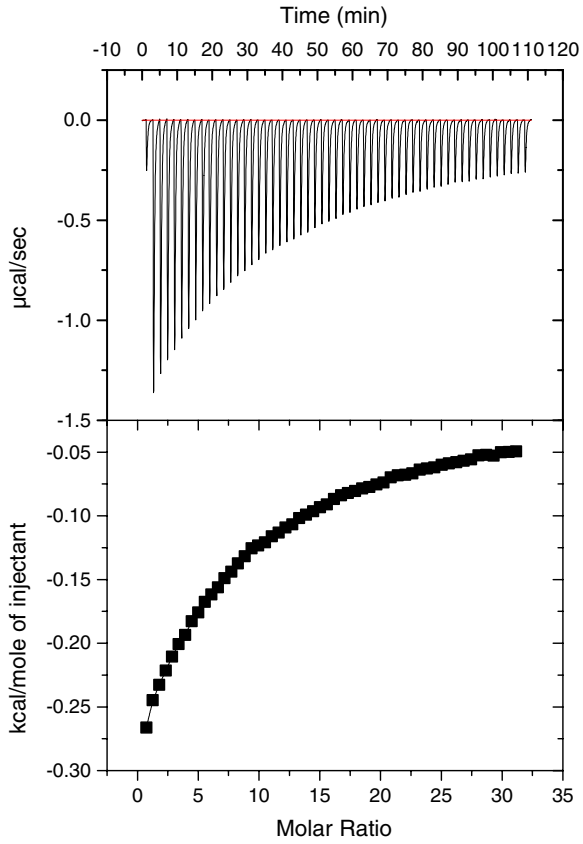

Fig. 4. ITC results for ConA titrations at 20 °C. fitted to a single binding site model 0.092 mM ConA titrated with 27.8 mM D-glucose. Parameter values based on the average of two measurements. Number of binding sites per monomer (n) 0.965 ± 0.01, Association constant (K_a) $= 205 \pm 5\ M^{-1}$, Binding energy (ΔH) 3300 ± 37 kcal mol^{-1}.

In addition to specific permeability changes in response to competitor concentration, changes in both pH and ionic strength are also likely to influence response. In Fig. 6 lysozyme concentration/time curves are shown as a function of pH over the range 2–9 (ionic strength was to 0.1 M using NaCl for each value). The inset gives rate as a function of pH showing a curve with 2 maxima.

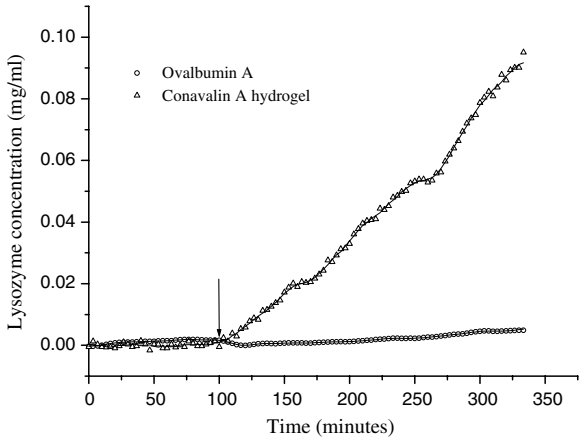

Fig. 5. Comparison of lysozyme diffusion through an ovalbumin and a ConA hydrogel. Experiments were conducted at 25 °C, 0.1 mol glucose was added at 100 min.

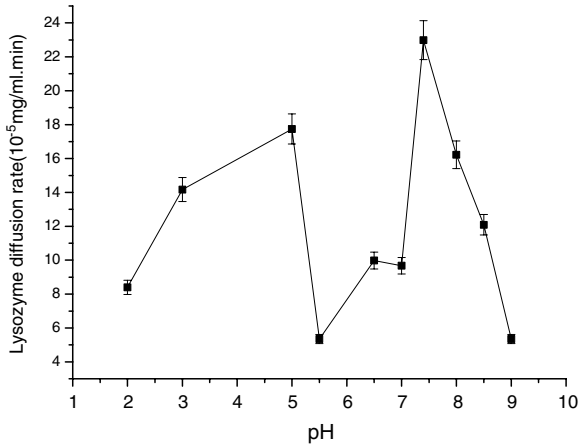

Fig. 6. The effect of pH on the diffusion of lysozyme through a ConA hydrogel. The lysozyme feed concentration was 2.0 mg/ml and the temperature 25 °C. 20 mM Phosphate buffer was employed throughout, with changes in ionic strength compensated by the addition of NaCl to maintain a value of 0.1 M.

Fig. 7 shows the effect of ionic strength on lysozyme transport at a fixed pH of 7.4. In this case transport rate increases between 0.05 and

Table 1
Summary of binding constants for ConA and ConA conjugates with dextran and with D- and L-glucose

Titrant	D-glucose	Dextran	L-glucose	D-glucose
Interaction	ConA with D glucose	ConA with dextran	ConA with L glucose	ConA with D-glucose in presence of dextran
K_a (M^{-1})	205 ± 5	31.7 ± 2	No binding detected	22 ± 2.2
Interaction	CM-dextran-ConA with D-glucose	CM-dextran-ConA with dextran	CM-dextran-ConA with L-glucose	CM-dextran-ConA with D-glucose in presence of dextran
K_a (M^{-1})	93 ± 3	3.9 ± 2	No binding detected	57.5 ± 3

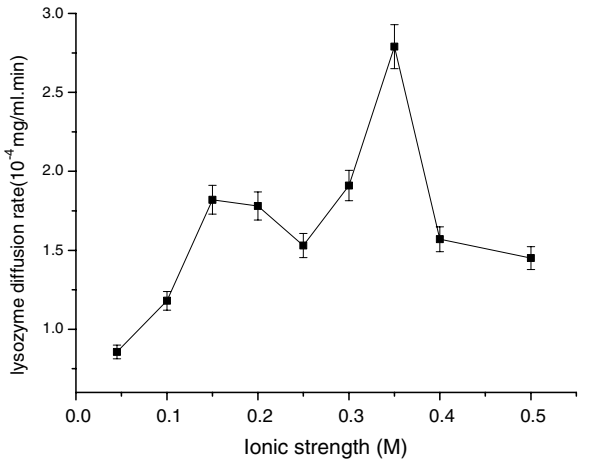

Fig. 7. The effect of ionic strength on the diffusion of lysozyme through a ConA hydrogel. The lysozyme feed concentration was 2.0 mg/ml and the temperature 25 °C. pH was maintained at 7.4 using 20 mM phosphate buffer. Ionic strength of the solution was varied by the addition of NaCl.

0.35 M and then decreases over the range 0.35–0.5 M.

The specificity of permeability changes in the membrane was determined using several solute molecules (D-glucose, L-glucose, glycerol and dextran). Fig. 8 shows that, as expected from the ITC results, D-glucose has the most pronounced effect, whereas L-glucose, glycerol and dextran show similar but less pronounced effects. In the case of L-glucose and dextran this would be anticipated from the ITC results. In control experiments, where lysozyme solution without carbohydrate/glycerol was used, little diffusion was observed. The response lag observed between addition of competitor and change in transport rate was approximately 20 min; this matches the residence time in the donor cell such that diffusion limitations will be partially masked during the time course of D-glucose build-up.

The results show that the hydrogel membrane allows lysozyme transport at a rate that is controllable using a soluble competitor molecule, and that this effect is specific to molecules recognized by the ConA receptor. This suggests that as anticipated, changes in permeability of the membrane where dextran serves as both a ligand for ConA affinity and a structural matrix, result from a reduction of internal affinity cross-links when a soluble competitor is introduced. It is also implicit in these results that a significant fraction of the total ConA is remains active during membrane synthesis.

An essential property of responsive gels of the type described here is the reversibility of the response generated. SEM results suggest that structural changes generated by the addition of competitor (D-glucose) are largely reversed when this is removed. In Fig. 9, the effect of sequential addition and removal of D-glucose on lysozyme transport is shown. As expected the reversible changes in physical properties are clearly mirrored by permeability changes in response to competitor concentration. The response shows that, while there is a diffusion time lag between each solution change, the introduction and removal of D-glucose causes a reversible switch between lower and higher diffusion rates.

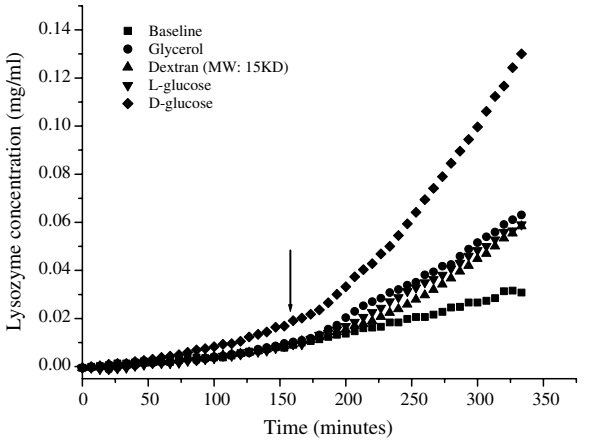

Fig. 8. The effect of various solutes on the diffusion of Insulin (2 mg/ml) through a ConA membrane (0.1 M D-glucose, L-glucose, dextran (12 kD) and glycerol). Solutes were added at 166 min. Measurements were conducted at 25 °C).

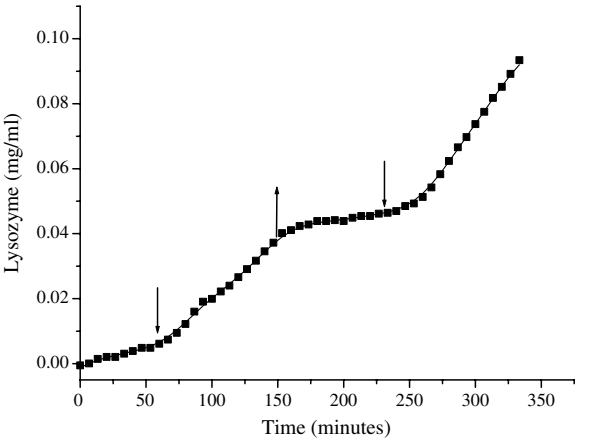

Fig. 9. Reversibility of insulin diffusion in response to changes in D-glucose. 0.1 M D-glucose plus insulin (2.0 mg/ml) buffer was added at the down-arrows. Insulin solution without glucose was restored at the up-arrows. Temperature 25 °C.

4. Discussion

Grafting carboxylic groups onto dextran using sodium chloroacetate while controlling reaction temperature and time, allows varying degrees of carboxylic substitution on the dextran backbone. These carboxylic groups can then be cross-linked to form hydrogels in the scheme shown in Fig. 1. Variation of the degree of substitution allows control of the residual number of non cross-linked carboxylic groups and hence the charge properties of the gels [24]. Incorporation of functional proteins into gels based on these carboxylated dextrans allows the formation of bio-responsive hydrogels gels with controlled charged properties. In this study, CM-dextran containing one COOH per 65 glucose residues was used to prepare gels incorporating protein. FTIR results support the proposed reaction scheme, but of greater significance is the evidence provided by the SEM results that demonstrate that the gels prepared via this route show reversible, biospecific structural changes. From the SEM results, it is apparent that hydrogel structure changes following exposure to different stimuli. The pictures suggest that the gel contains voids of around 1 μm surrounded by thin walls of polymer. When the gels shrink, both void dimensions and wall thickness are reduced. The ability of the gels to controllably exclude proteins suggest that these voids are not contiguous and that transport is determined by the gel layers comprising the void walls. The effects of the different competitors are as might be expected from the specificity of ConA. While both D-glucose and dextran lead to morphology changes that correlate with increases in gel permeability, L-glucose has little observable effect. These results show that at a structural level the effect of competitor is both specific and reversible.

To achieve a functional gel it is essential that the protein is not inactivated during the coupling process. The SEM results provide indirect evidence that this is the case, but they do not eliminate the possibility that structural changes are an artefact resulting from effects unrelated to biospecific interactions. However, the fundamental investigation of the binding interactions anticipated using ITC confirm that the ConA coupled gel precursors maintain their ability to bind D-glucose and glucose-based polymers (dextran). It is interesting to note that the binding affinity of CM-dextran-ConA for dextran is lower than that observed for native ConA, possibly as a result of the carboxylic groups complexing the divalent cations required for ConA binding (Ca^{2+} and Mn^{2+}). For some applications (e.g., affinity adsorption) this might cause a problem. However, for use in a responsive polymer weaker binding, indicative of a faster dissociation rate, is likely to have a beneficial effect in reducing response times.

In addition to changes in the concentration of specific competitors, responsive gels are also likely to experience changes in both pH and ionic strength. These effects are shown in Fig. 6. Previous studies have shown that the carboxylic groups in these gels have a dissociation constant of 6.1 while lysozyme has an isoelectric point of 11. This suggests that at pH values below 5 the gel will be carry a small positive charge while the lysozyme will be positively charged; this will lead to charge repulsion effects limiting transport. As the pH is increased towards 5 the positive charge on the gel will decrease and transport rate increases. Above 5 the concentration of negative charges on the gel starts to increase leading to ion exchange interactions between protein and gel, again limiting transport. In this region, transport properties might also be influenced if the immobilized ConA switches between a dimer and tetramer conformation as would be expected in free solution. As pH is further increased the ion exchange effect is lost as the positive charge on the lysozyme starts to decrease leading to the second transport maximum. Also, as the carboxylic groups dissociate and the net negative charge in the gel increases, charge repulsion effects will cause the gel to swell, increasing permeability. Further increases in pH lead to an increase in the negative charge on the lysozyme again leading to charge repulsion limitations with the negatively charged gel.

The effect of ionic strength was measured at pH 7.4 (Fig. 7) where it is likely that transport will be limited by ion exchange interactions between gel and lysozyme. Therefore, as ionic strength is increased, swamping these effects, transport rate increases. Further increases in ionic strength result in a degree of gel shrinkage leading to a subsequent decrease in transport rate.

The key requirements of the gel are specific and reversible changes in transport properties in response to changes in competitor concentration. The results reported here confirm that, as expected from the SEM results, increases in permeability are observed in response to changes in D-glucose concentration, and that these effects are reversible over a number of addition/removal cycles.

5. Conclusion

A simple procedure is reported for the synthesis of D-glucose responsive hydrogels. This allows production of gels with varying levels of protein substitution and with a controllable degree of carboxylic group substitution.

There are two major advantages of the materials described here compared with the glucose responsive gel we described previously [22]: (i) The gel no longer requires the use of a potentially cytotoxic triazine group, and hence is more likely to be biocompatible. (ii) The use of CM-dextran allows the intrinsic charge properties of the gel to be easily controlled such that gels can be tailored use in environments of different pH and ionic strengths.

In the course of these studies it was found that the gelation time and the mechanical strength of the final gel produced are dictated by the initial degree of substitution of carboxylic groups and subsequently by the concentration of coupled protein e.g. the gelation time for hydrogel-grafted ConA is far shorter than that of gels without protein produced using the same procedure (10 min rather than 50). This suggests that mechanical properties can be modified independently of response levels by including inert proteins or other amine containing compounds to increase the degree of covalent cross-linking.

Acknowledgement

We gratefully acknowledge financial support from the BBSRC Grant No. 86/E12129.

References

[1] S. Sershen, J. West, Adv. Drug Deliv. Rev. 54 (2002) 1225.
[2] T. Miyata, T. Uragami, K. Nakamae, Adv. Drug Deliv. Rev. 54 (2002) 79.
[3] I.J. Goldstein, in: H. Bittiger, H.P. Schnebli (Eds.), Concanavalin A as a Tool, Wiley, London, 1976, pp. 55–65.
[4] I.J. Goldstein, L. So, Arch. Biochem. Biophys. (1965) 111.
[5] I.J. Goldstein, C.M. Reichert, A. Misaki, P.A. Gorin, Biochem. Biophys. Acta 317 (1973) 500.
[6] M. Brownlee, A. Cerami, Science 206 (1979) 1190.
[7] S.W. Kim, H.A. Jacobs, Drug Dev. Ind. Pharm. 20 (1994) 575.
[8] F. Liu, S.C. Song, D. Mix, M. Baudys, S.W. Kim, Bioconjugate Chem. 8 (1997) 664.
[9] M.J. Taylor, Patent Application No. WO 95/01186 June 30, 1993.
[10] M.J. Taylor, S. Tanna, J. Pharm. Pharmacol. 46 (Suppl. 2) (1994) 1051.
[11] M.J. Taylor, S. Tanna, P.M. Taylor, G. Adams, J. Drug Target. 3 (1995) 209.
[12] S. Tanna, M.J. Taylor, J. Pharm. Pharmacol. 46 (Suppl. 2) (1994) 1051.
[13] S. Tanna, M.J. Taylor, J. Pharm. Pharmacol. 49 (Suppl. 4) (1997) 76.
[14] S. Tanna, M.J. Taylor, Pharm Pharmacol. Commun. 4 (1998) 117.
[15] S. Tanna, M.J. Taylor, Proc. Int. Symp. Contr. Rel. Bioact. Mater. 25 (1998) 737B.
[16] S. Tanna, M.J. Taylor, G. Adams, J. Pharm. Pharmacol. 51 (10) (1999) 1093.
[17] S. Tanna, T.S. Sahota, J. Clark, M.J. Taylor, J. Drug Target. 10 (2002) 411.
[18] S. Tanna, T. Sahota, J. Clark, M.J. Taylor, J. Pharm. Pharmacol. 54 (11) (2002) 1461.
[19] S.J. Lee, K. Park, Proc. Int. Symp. Contr. Rel. Bioact. Mater. 21 (1994) 93.
[20] A. Obaidat, K. Park, Pharm. Res. 13 (1996) 989.
[21] A. Obaidat, K. Park, Biomaterials 18 (1997) 801.
[22] M. Tang, R. Zhang, A. Bowyer, R. Eisenthal, J. Hubble, Biotechnol. Bioeng. 82 (1) (2003) 47.
[23] T. Miyata, N. Asami, T. Uragami, Nature 399 (1999) 766.
[24] R. Zhang, M. Tang, A. Bowyer, R. Eisenthal, J. Hubble, Biomaterials 26 (2005) 4677.
[25] S. Benslimane, R. Guidoin, D. Marceau, Y. Merhi, M.W. King, M.F. Sigo-Luizard, Eur. Surg. Res. 20 (1988) 18.
[26] K. Tomihata, Y. Ikada, J. Biomed. Mater. Res. 37 (1997) 243.
[27] J.M. Tedder, A. Nechvatal, A.W. Murray, J. Carnduff, Basic Organic Chemistry, Wiley, London, 1972.
[28] Z. Grabarek, J. Gergely, Anal. Biochem. 185 (1990) 131.
[29] G. Socrates, Infrared Characteristic Group Frequencies: Tables and Charts, second ed., Wiley, London, 1997.