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A novel pH- and ionic-strength-sensitive carboxy methyl dextran hydrogel

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Abstract

A fast and simple method for the preparation of pH-sensitive hydrogel membranes for drug delivery and tissue engineering applications has been developed using carbodiimide chemistry. The hydrogels were formed by the intermolecular cross-linking of carboxymethyl dextran (CM-dextran) using 1-ethyl-(3-3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). Infrared spectra of the hydrogels suggest the formation of ester bonds between the hydroxyl and carboxyl groups in the CM-dextran. The porosity of the hydrogels produced, as shown by protein diffusion, increases in response to changes in the pH and the ionic strength of the external medium. The results show pH-dependent swelling behaviour arising from the acidic pedant groups in the polymer network. The diffusion of the protein lysozyme through the hydrogel membranes increased with increases in both pH (5.0–9.0) and ionic strength. The effect of changes of pH and ionic strength on the hydrogel's permeability was shown to be reversible. Scanning electron microscopy of these hydrogels showed that pH-dependent changes in permeability are mirrored by morphological changes in gel structure.

Keywords: Dextran; Drug delivery; Hydrogel; Membrane; Responsive

1. Background

Stimuli-responsive hydrogels have been produced that exhibit dramatic changes in their swelling behaviour, network structure, permeability and mechanical strength in response to a number of external stimuli, including pH, ionic strength of the surrounding fluid, temperature, presence of specific solutes and applied electrical or magnetic fields [1]. Because of their nature, these materials can be used in a wide variety of applications, including separation, biosensors, drug delivery devices and tissue engineering [2].

pH-sensitive polymers are produced by adding pendant acidic or basic functional groups to the polymer backbone; these either accept or release protons in response to appropriate pH and ionic strength changes in aqueous media [3]. The network porosity of these hydrogels will change with electrostatic repulsion. For example, ionic hydrogels containing carboxylic or sulphonic acid groups show either sudden or gradual changes in their dynamic and equilibrium swelling behaviour as a result of changing the external pH [4,5]. The degree of ionisation of these hydrogels depends on the number of pendant acidic groups in the hydrogel, which results in increased electrostatic repulsions between negatively charged carboxyl groups on different chains. This, in turn, results in an increased hydrophilicity of the network, and greater swelling ratios at high pH. Conversely, hydrogels containing basic pendant groups, such as amines, ionise and show electrostatic repulsion at low pH.

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Hydrogels currently used, or with potential applications in drug delivery or tissue engineering, are divided into two categories according to their natural or synthetic origin. Hydrogels from various synthetic polymers have been widely investigated by using neutral co-monomers, such as 2-hydroxyethyl methacrylate, methyl methacrylate and maleic anhydride [6–9]. While considerable effort has been made to synthesise and characterise acrylate-based ionic hydrogels, these materials are not biologically degradable by either hydrolytic or enzymatic mechanisms. As a result, acrylate systems are limited in their potential as biodegradable drug-delivery platforms. To overcome this liability, a range of natural polymers has been used to prepare cross-linked hydrogel networks. For example, pH-sensitive hydrogels based on polypeptides, proteins and polysaccharides have all been produced [10,11].

However, limitations of hydrogels from natural polymers have motivated approaches to modify these polymers, as well as to use various synthetic polymers. Dextran is a polysaccharide mainly composed of 1,6-linked D-glucopyranose residues. Owing to their low tissue toxicity and high enzymatic degradability, dextran hydrogels have been frequently considered as a potential matrix system for drug delivery or controlled release of bioactive agents [12]. Several approaches to the preparation of dextran hydrogels have been adopted. Hydrogels have been prepared directly by cross-linking dextran with either 1,6-hexanediisocyanate or glutaraldehyde [13,14]. Sequential reactions of dextran with glycidyl acrylate, followed by polymerisation of acrylated dextran have also led to the formation of a polymer network [15]. Methacrylation of dextran has been conducted with full control of the degree of substitution by transesterification of glycidyl methacrylate with dextran in dimethyl sulphoxide [16,17]. Kim and Chu [18] and Kim et al. [19] obtained dextran hydrogels by UV irradiation of methacrylated and acrylated dextrans that were synthesised by reacting dextran with methacrylic anhydride, and then bromoacetyl bromide and sodium acrylate. Recently, Chiu et al. [12] have reported dextran hydrogels prepared by radical copolymerisation of methacrylate–dextran with acetic anhydride in borate buffer at room temperature, using ammonium peroxydisulphate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine as an initiation system.

We recently reported the synthesis of a D-glucose-sensitive dextran hydrogel containing concanavalin A and demonstrated that the diffusion of proteins (insulin, lysozyme, and BSA) through this gel varied with D-glucose concentration [20]. We have also shown that hydrogels synthesised by grafting Cibacron Blue and lysozyme to dextran can yield a reversible and specific controlled diffusion of both cytochrome C and haemoglobin in response to changes in environmental NAD concentration [21]. Here we report the synthesis of a novel pH-responsive dextran hydrogel produced by the intermolecular cross-linking of carboxymethyl dextran (CM-dextran) using 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). EDC was used as it is not incorporated into the cross-linked structure, but is simply changed to a water-soluble urea derivative, which can subsequently be washed out. The cytotoxicity of the urea derivative has been found to be low compared with that of EDC [22]. The hydrogel is formed by ester bonds between hydroxyl and carboxyl in CM-dextran in the presence of EDC and NHS. In this respect the synthesis method is similar to that of Tomihata and Ikada [23] who synthesised an uncharged hydrogel for tissue engineering applications. Our primary targets here were to demonstrate that pH-sensitive hydrogel membranes could be produced by including an excess of carboxylic groups, that these changed their porosity reversibly in response to changes in environmental pH, and that this response could control the diffusive transport of a protein (lysozyme) though the membrane.

2. Materials

Dextran and lysozyme were obtained from Sigma-Aldrich, UK. All other chemicals were of reagent grade and obtained from Lancaster Synthesis Ltd., UK.

3. Methods

3.1. Gel synthesis

Carbodiimide chemistry was employed to cross-link carboxylic groups and hydroxyl groups in CM-dextran. According to the expected cross-linking mechanism, EDC can react with the carboxyl groups in CM-dextran to form an activated *O*-urea.

The synthesis procedure was as follows, and is summarised in Fig. 1: 5 g 480 kD dextran was dissolved in 75 ml of distilled water, followed by the addition of 5 g sodium chloroacetate. The carboxymethylation reaction was initiated by the addition of 25 ml of 8 M NaOH and the reaction mixture was diluted to 100 ml total volume. Carboxymethylation was allowed to proceed for 15 min at 62 °C to introduce sufficient groups for cross-linking a non-pH-sensitive hydrogel membrane. More highly substituted materials for the preparation of pH-sensitive gels were incubated at 70 °C for 1 h. Reaction mixtures were stirred throughout. The reaction was terminated by lowering the solution pH to 7 with 6 M HCl. The product was then precipitated with 300 ml absolute ethanol and allowed to stand overnight. The precipitate was dissolved in distilled water and exhaustively dialysed against distilled water before freezing and lyophilisation to give a white powder.

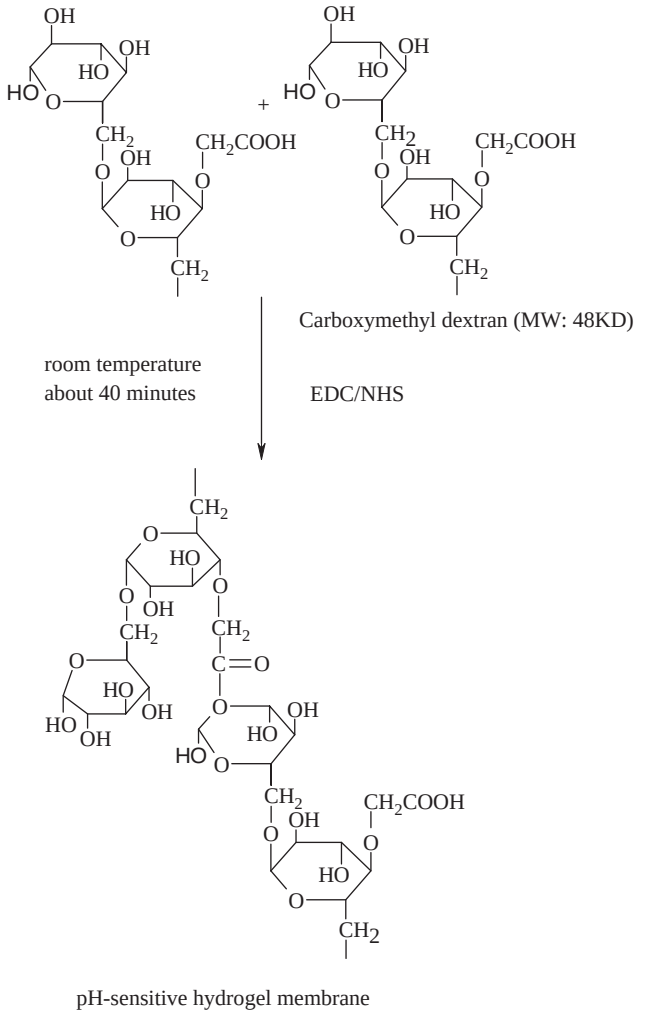

Fig. 1. Gel synthesis protocol.

The number of COOH groups in the two CM-dextrans preparations (non-sensitive and sensitive) were calculated by means of acid titration. The ratio of COOH groups per dextran was 1-COOH/13 glucose units for the material used for the pH-sensitive hydrogel and 1-COOH/63 glucose units for the non-pH-sensitive hydrogel.

To make the hydrogel membranes, 0.5 g CM-dextran was dissolved in 3 ml distilled water while stirring thoroughly. EDC (160 mg) and NHS (25 mg) were dissolved in 1 ml distilled water. Once any gas bubbles had been removed, 1 ml of the EDC/NHS mixture was added and stirred for 30 min. The solution was then cast on nylon gauze for mechanical support (pore size 0.1 mm, thickness 0.05 mm) between two glass plates using spacers to give the required total membrane thickness.

3.2. Gel characterisation

Infrared spectroscopy was performed on a Bruker-equinox 55 FT-IR spectrometer. 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} with a resolution of 2 cm^{-1}.

3.3. pH effects on gel morphology

The morphology of the hydrogel incubated at various pH was examined using a Jeol 6310 SEM equipped with a cryo-stage and energy-dispersive X-ray (EDX). A sample of the hydrogel was clamped between two pieces of aluminium sheet and rapidly frozen in liquid nitrogen. It was then introduced into the SEM-chamber pre-cooled to a temperature of ca. $-160\,^{\circ}C$. The stage was then heated to a temperature of ca. $-80\,^{\circ}C$ to sublimate off the surface water. After cooling to $-160\,^{\circ}C$, the sample was gold sputtered for 3 min. The sample was scanned at a magnification of $2000\times$. It was not possible to prepare a sample of gel exposed to pH values >8.0 as the sample disintegrates under the conditions of preparation for SEM.

3.4. Gel transport measurements

The trans-membrane transport of lysozyme as a model protein was studied using a diffusion cell consisting of donor and receptor chambers of equal volumes of 4.4 ml, as described by Tang et al. [20]. Hydrogel membranes 0.5 mm thick, 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 buffer. The donor chamber was connected to the lysozyme reservoir via a pump. The receptor chamber was connected to a UV–visible spectrophotometer to allow lysozyme diffusion across the membrane to be automatically monitored and logged from optical density changes. Lysozyme diffusion from donor to receptor chambers was calculated using calibration data for lysozyme at 280 nm. The effect of pH and ionic strength was investigated by varying the buffer composition of the lysozyme solution fed to the donor chamber and following the resultant changes in transport rate. The results shown in Figs. 4–7 are representative of experiments repeated at least three times. The transport studies involving pH variation were conducted at a fixed ionic strength 0.1 M. Those involving ionic strength variation were performed at pH 5.5 and 7.4. All transport studies were at carried out at $25\,^{\circ}C$. Buffers were based on potassium phosphate and ionic strength was adjusted using NaCl.

4. Results

4.1. Gel characterization

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 1580 cm^{-1} as shown in Fig. 2. This indicates that activated carboxyl groups can react with hydroxyl groups in CM-dextran to form ester linkages. A new peak at just below 1800 cm^{-1} indicates formation of the ester linkage during gel formation.

4.2. pH effects on gel morphology

The SEM revealed the cross-sectional interior structure of swollen hydrogels equilibrated in buffer at pH 7.4 and 5.5. Two different structures were observed as shown in Figs. 3a and b. The hydrogel macrostructure at pH 5.5 is seen to be compact compared with that in pH 7.4. Fig. 3c also illustrates that this effect is reversible.

4.3. Gel transport measurements

4.3.1. Reversibility of the pH response

The reversibility of the pH effect was studied in diffusion experiments as described in Section 3. By switching between lysozyme solutions in pH 5.5 and 7.4 buffer (20 mM) while maintaining a constant ionic strength of 0.1 M it was possible to determine the diffusion of the protein through the membrane as the conditions are successively alternated. In the experiment shown in Fig. 4, lysozyme (2.0 mg/ml in a 100 ml reservoir) was used for the first 200 min at pH 5.5, providing the baseline diffusion curve. At 200 min, the pH 5.5 solution was replaced by protein solution at pH 7.4. As the total amounts of lysozyme 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 constant throughout the run. A significant increase in the rate of lysozyme diffusion occurred about 20 min after the switch to the high-pH solution. This cycle was repeated: at 350 min, protein solution in pH 5.5 was reintroduced. The curve of diffusion became stable after 50 min. Repetition of this procedure gave a similar response. The results show that the membrane exhibits a reversible response to pH

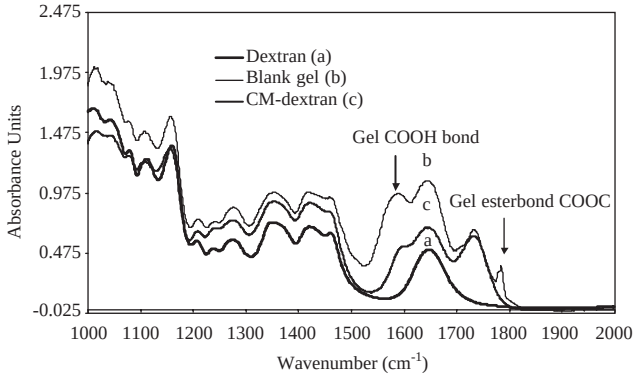

Fig. 2. Dextran hydrogel characterised by FT-IR.

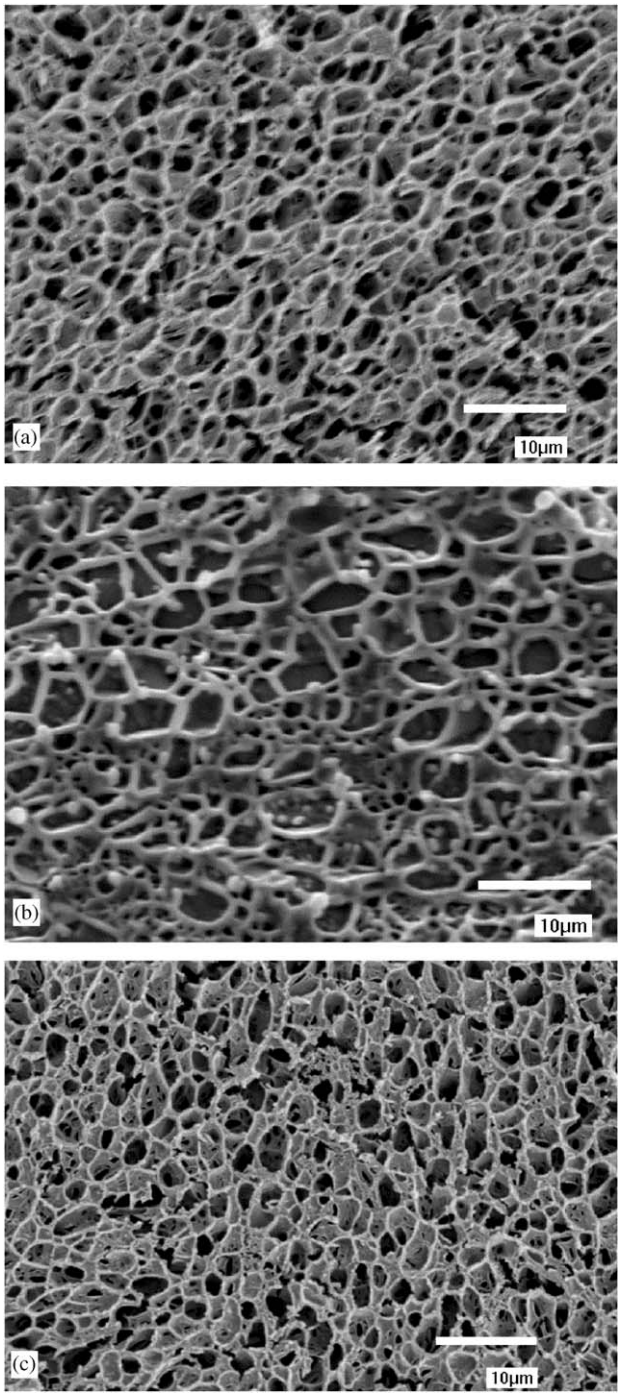

Fig. 3. SEM showing structures of hydrogels in buffer at: (a) pH 5.0; (b) pH 7.4; (c) pH 5.0 after exposure to pH 7.4 buffer.

change under conditions of constant ionic strength (0.1 M). This effect of pH could be observed over a 12 h period involving up to six switches of pH.

4.3.2. Effect of pH on diffusion rate

The diffusion rate of protein across the hydrogel membrane was measured in response to pH changes by adjusting the pH of the 20 mM buffer while compensating

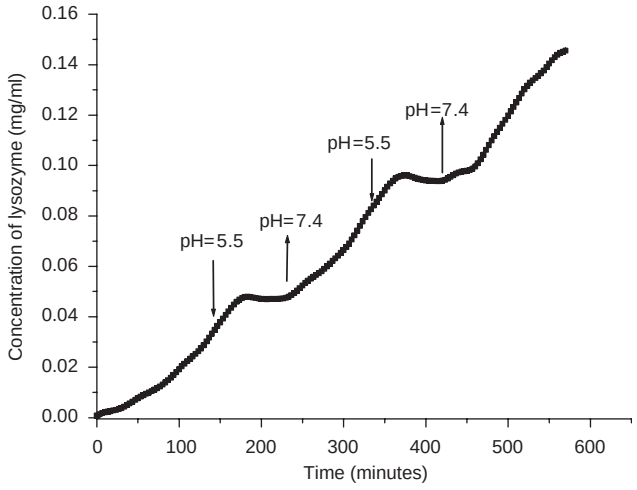

Fig. 4. Reversibility of transport through the hydrogel in response to pH change. Initial pH 7.4. Up and down arrows indicate points at which the buffer pH was switched: ↓ to pH 5.5; ↑ to pH 7.4.

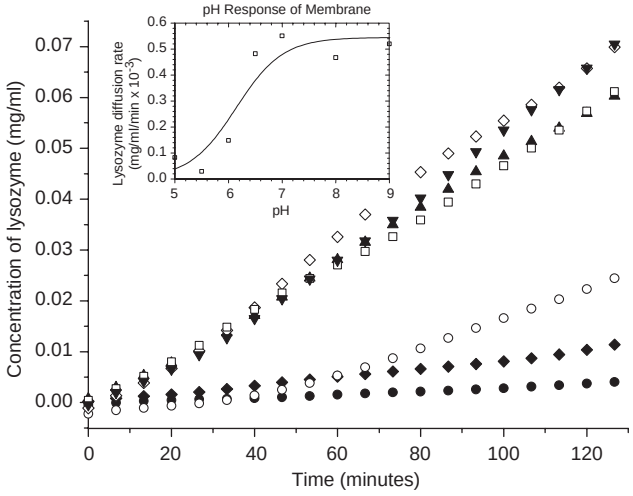

Fig. 5. Effect of pH on lysozyme diffusion using a pH responsive hydrogel. pH values: ♦ 5.0, ● 5.5, ○ 6.0, ▲ 6.5, ◇ 7.0, □ 8.0, ▼ 9.0. Inset: plot of lysozyme diffusion rate against pH; the solid line is a fit of a titration curve to the data points.

for consequential changes in ionic strength by the addition of sodium chloride to maintain an ionic strength as 0.1 M throughout. Fig. 5 shows lysozyme transport through the membrane over a pH range of 5.0–9.0 with all experiments conducted using the same membrane. To avoid artefacts arising from diffusion lags after pH changes a pre-incubation period of 60 s was allowed before data collection took place. The results show that the transport rate of lysozyme across the hydrogel increases as the pH is increased and the acidic moieties of the CM-dextran become increasingly ionised. Similar results have been observed with pH-sensitive polypeptide hydrogels, methacrylated dextran hydrogels, polyacrylamide-*g*-guar gum microgels and chitosan–polyvinyl pyrrolidone hydrogels [11,12,24,25]. We therefore assume that in the pH-sensitive hydrogel grafted carboxyl groups act as polyvalent weak acids. At high pH, the COOH groups dissociate inducing electrostatic repulsion. As a consequence, the distance between chains increases and the hydrogel structure becomes more permeable to large molecules. The inset curve in Fig. 5 shows that the diffusion-rate/pH-response fits to a titration curve giving a pKa value of 6.1.

4.3.3. Effect of ionic strength on diffusion rate

The ionic strength characteristics of the hydrogels were investigated by measuring protein diffusion over the ionic strengths range 0.045–0.3 M at pH values of 5.5 and 7.4. Considering the hydrogel as a polyelectrolyte suggests that porosity should decrease as ionic strength increases. As shown in Fig. 6, at pH 7.4 the diffusion rate of lysozyme first increased with increasing ionic strength up to 0.15 M; however, above this value it decreased with further increases in ionic strengths. A similar result was attained at pH 5.5, with a shift of ionic strength to 0.2 M at which the diffusion rate

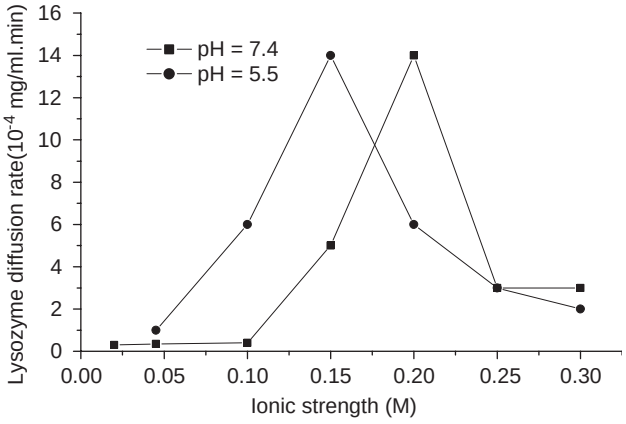

Fig. 6. The rate of lysozyme diffusion rate as a function of ionic strength at pH 7.4 and 5.5.

reaches its maximal point. That this phenomenon is reversible is confirmed by the observation that identical results were obtained when ionic strength was decreased over the measured range. Ju et al. [26] have also reported similar results using alginate/PNIPAAm-NH_2 graft hydrogels.

4.3.4. Osmotic pressure effects

As changes in ionic strength generated using sodium chloride will also influence osmotic pressure, the effects of osmotic pressure were assessed using a non-electrolyte. Diffusion experiments were conducted using a 0.5 mm membrane with different osmotic pressures achieved by varying sucrose concentrations in 20 mM buffer pH 7.4 ($I = 0.1$ M). Sucrose solutions of 0.2 and 0.5 M were introduced after 100 min. In both cases there was no observable effect on transport rate, suggesting that the diffusion-rate fluctuation seen with changes in

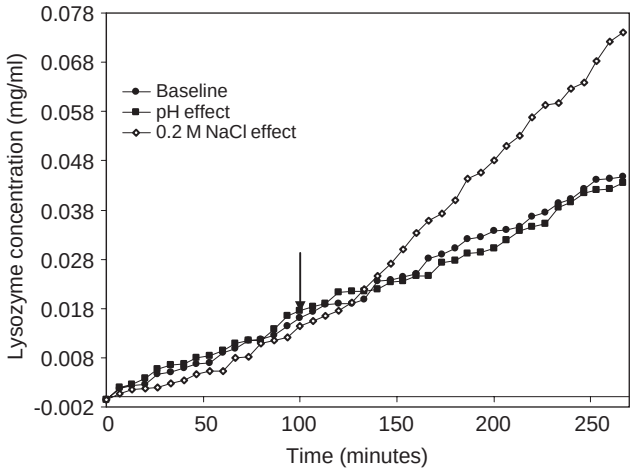

Fig. 7. The pH and ionic strength effect on 2 mg/ml lysozyme through the non-pH-sensitive hydrogel membrane. The system was initially operated in pH 5.5 buffer. At 100 min (↓ on graph), the buffer was changed to either pH 7.4 buffer 20 mM (ionic strength 0.1 M) or pH 5.5 buffer (ionic strength 0.2 M). The baseline was recorded at pH 7.4, ionic strength 0.1 M.

pH and salt concentration are not the result of changes in osmotic pressure.

4.3.5. pH and ionic strength effects on control hydrogels

The proposed response mechanism of the pH-sensitive hydrogel membrane is based on the presence of underivatised COOH groups in the hydrogel. The permeability of the hydrogel membrane only increases when these groups are ionised under conditions of high pH. In contrast, hydrogels produced from dextran with a lower carboxylic acid substitution where most of the groups will be involved in the cross-linking reaction would not be expected to show a significant pH response. From the curve in Fig. 7, which shows the diffusion rate of lysozyme across a hydrogel made from a CM-dextran with a substitution ratio 1 COOH per 65 glucose units, it is apparent that there is no change in diffusion rate as external pH is increased from 5.5 to 7.4. However, ionic strength effects are still apparent when 0.2 M NaCl is added to the buffer used.

5. Discussion

pH-sensitive hydrogels were successfully prepared by chemical cross-linking of CM-dextran using EDC and NHS. These cross-linking agents introduce 'zero length' ester-cross-links between carboxylic acid groups and hydroxyl groups in the CM-dextran. When compared with commonly used methacrylated dextran hydrogels, prepared by radical polymerisation of methacrylated dextran in the presence of cross-linking agents, hydrogels based on CM-dextran with EDC and NHS have several advantages. Firstly, the preparation procedure is simple and rapid. More importantly, variation of carboxylic group density and cross-linking reagent concentration allow control of both charge density and degree of cross-linking in the hydrogel so that the balance between degradation rates and mechanical properties can be easily controlled. Finally, EDC/NHS is not incorporated into the hydrogel, in contrast with conventional cross-linking agents [27], instead reacting to form a water-soluble urea derivative, which has far lower cytotoxicity and is easily washed out.

The formation of an ester bond as the cross-link bridge was confirmed by FT-IR. It is obvious that the chemically reactive groups responsible for the cross-linking of CM-dextran molecules must be hydroxyl and carboxyl from Fig. 1. Evidence for the presence of COOH groups in the hydrogel is also provided by the data obtained from FT-IR measurements.

A prominent transition of the hydrogel structure in response to pH at the macro-level is shown in Fig. 3. Although the size of pores in the hydrogel as shown by the SEM is much larger than the diameter of the protein used in subsequent diffusion studies, we assume that this reflects a similar effect at the molecular level in terms of polymer structure. Thus, the functional (i.e. transport) properties of the gel stem from changes in the polymer, where ionic components will produce alterations in electrostatic repulsion with pH change.

It is the COOH substitution levels in the hydrogel that determine whether the hydrogel is sensitive to pH. As seen in Fig. 7, the hydrogel will lose sensitivity to external pH change when there are few or no COOH groups in the hydrogel. It follows that the sensitivity of this hydrogel can be tailored for a specific application by varying the degree of carboxymethyl substitution in the dextran used for its preparation.

In terms of potential applications for a gel of this type, the most important finding is that the effect of pH on permeability is reversible as shown in Fig. 4. The lag time in part results from dead times in the flow system but is also dependent on the movement in the polymer chains, which comprise the gel structure. This explains the a shorter lag time for the increase in diffusion in response to pH 7.4 compared with the lag time for the decrease in response to pH 5.5. This difference between expansion and collapse rates has also been reported by Hassan et al. [28] in swelling studies conducted with methacrylate-based pH-sensitive gels.

The diffusion data for lysozyme across the hydrogel at different pH or ionic strengths shows the porosity response of the hydrogel to its external environment. The results in Fig. 5 indicate that increasing pH from 5.5 to 7.4 results in a considerable increase in diffusion rate. The apparent pKa of 6.1 observed for the CM-dextran, as determined by titration is in good agreement with the value observed for the midpoint of the plot of diffusion rates against pH shown in Fig. 5 (inset).

Regarding the effect of ionic strength, the dissociation of COOH in CM-dextran may be enhanced as the ionic strength increases up to a fixed value; but with ionic strength continuously increasing, the anionic groups in the hydrogel are screened by Na^+ ions. As a result, the conformation in the hydrogel changes from an expanded to a more compact matrix, so that the diffusion rate drops with increasing ionic strength. Another possible contribution to this result is that chloride ions present in the external solution might have swamped the negatively charged carboxylic group as described by Soppimath et al. [25].

6. Conclusions

A dextran-based hydrogel incorporating carboxyl has been prepared using carbodiimide chemistry and cast into membranes. The diffusion rates of proteins through the membranes is influenced by pH, increasing as the pH is raised, due to loosening of the gel structure resulting from mutual repulsion of ionised carboxyl groups. Protein diffusion rates through the hydrogel membranes are also sensitive to changes in ionic strength. This response appears as a bell-shaped curve, increasing at low ionic strengths, and then decreasing as ionic effects swamp the charge repulsion effects.

Acknowledgements

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