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A Smart Membrane Based on an Antigen-Responsive Hydrogel

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ABSTRACT: Hydrogel membranes have been fabricated that incorporate antibody/antigen moieties. The permeability of large solutes through these membranes is dependent on the presence of soluble antigen that can compete with the internal interactions between antibody and antigen leading to an increase in gel mesh size. Specifically, the membrane's structure is based on a dextran backbone grafted with a fluorescein isothiocyanate (FITC) antigen and a sheep anti-FITC IgG antibody. The backbone is covalently cross-linked by conjugated divinyl sulfone (DVS) groups. The gel structure is additionally stabilized by affinity crosslinks formed by biospecific interactions between the bound IgG and FITC. FTIR spectra of the gel are consistent with formation of covalent bonds between cysteine groups in the IgG and DVS groups in the dextran. Results obtained using isothermal titration calorimetry (ITC) confirmed the competitive interaction binding between IgG-FITC-dextran and free sodium fluorescein at pH 5.0. Scanning electron microscopy (SEM) of samples prepared using cryofixation and cryofracturing techniques showed that observed changes in permeability correlate with free fluorescein-dependent structural changes in the gel. Three-dimensional images obtained from confocal laser scanning microscopy show that these changes occur throughout the gel and indicate that SEM results are not artifacts of sample preparation. The permeability of these gels, as shown by blue-dextran (12 kDa) diffusion, increases in response to the presence of free fluorescein of the external medium, which causes competitive displacement of the affinity cross-links. Sequential addition and removal of sodium fluorescein showed that these permeability changes are reversible.

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KEYWORDS: gel; matrix; affinity; biospecific; permeability; mesh

Introduction

Hydrogels are three-dimensional networks of polymers that absorb and retain a large amount of water. The equilibrium swelling ratio is an important parameter of a gel as it determines the water content of the gel at equilibrium (Peppas and Wright, 1996). In hydrogels the chains are cross-linked to prevent dissolution of the gel the aqueous medium. The chains can be held together by covalent bonds, hydrogen bonds, van der Waals' interactions, or ionic interactions (Kamath and Park, 1993). Hydrogels have been developed that alter their structure in response to environmental factors. These are sometimes termed "smart" or responsive hydrogels. Those that are responsive to biological molecules act as biosensors and can detect alterations in environmental conditions. Both physical and chemical stimuli have also been used to induce changes in such hydrogels. Physical stimuli include temperature, pressure, sound, magnetic field, and electric fields (Dinarvand and Emanuele, 1995; Suzuki et al., 1996; Zhong et al., 1996). Chemical stimuli include pH, specific ions, and biomolecules; these include glucose and various proteins.

Many biosystems use negative or positive feedback mechanisms to detect specific ions or biological molecules and induce conformational changes or rearrangement of biological molecules to elicit a biological function (an effector response). For example, a complex homeostatic control system functions to maintain the body's core temperature. The system continuously monitors the temperature of the body and triggers a variety of responses based on the sensing inputs. Responsive-hydrogels have the potential to mimic some of these natural feedback mechanisms as they can respond to the presence of an effector directly as it induces intrinsic structural changes. This response can be used to develop "smart" systems that

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could have many potential applications in the fields of biotechnology and medicine (Miyata et al., 2002). For example, glucose-responsive hydrogels have been synthesized which are based on cross-linked hydrophilic polymers (Hassan et al., 1997).

The specific chemo-responsive hydrogels described herein are designed to contain internal non-covalent interactions based on pendant ligands and complementary receptors. These cross-links can be broken by soluble ligand or receptor competitors, which can diffuse into the gel and displace the internal affinity cross-links. Subsequent non-specific diffusion of solutes through the membrane depends on the size and shape of the mesh pores and on the extent of the interactions between the competitive interactions between a specific molecule and the internal affinity links in the polymer.

The degree of cross-linking determines the pore size and porosity of the gel and therefore dictates its molecular-size exclusion properties. In hydrogels synthesized by Tang et al. (2003), two dextrans of different molecular weights are coupled, the smaller dextran having covalently grafted concanavalin A (ConA). Coupling of this to the larger dextran allows control over the total amount of ConA in the material. The affinity of ConA for glucose units on the dextran backbone provides additional affinity cross-links which are competitively inhibited by free glucose. This results in a decreased number of total cross-links in the presence of glucose, making the membrane more permeable to small proteins such as insulin. The principle of using competitors of internal affinity cross-links as a permeability switch is a general one. For example, based on earlier work (Mayes et al., 1990), Tang et al. (2004) have fabricated dextran hydrogel membranes with additional internal affinity interactions based on Cibacron blue/lysozyme. The permeability of these membranes to solute diffusion can be switched from a closed to an open state in the presence of free NAD, a competitive ligand of the internal affinity interactions.

Antibody and antigen interactions are highly specific, and in the gel described in this report, take the place of the glucose-ConA couple described above (Tang et al., 2003). Several approaches can be used to prepare such antigen-responsive hydrogels, including the physical trapping of the antibody or antigen in the network of the gel, the chemical conjugation of the antibody or antigen to the network, and the use of antibody–antigen pairs as reversible cross-linkers after chemically grafting them to the network (Lu et al., 1999, 2003).Lu et al. (2003) prepared antigen responsive hydrogels containing antibody Fab' fragments incorporated into the hydrogel network structure by the copolymerization of the polymerizable Fab' fragments with NIPAAm and N,N'-methylenebisacrylamide (MBAAm). The hydrogels demonstrated reversible volume changes in alternative incubations with fluorescein at pH 5.0 in acetate buffer, where the fluorescein was relatively hydrophobic since it was mainly present in acid (unionized) form. The binding of relatively large amounts of the hydrophobic fluorescein rendered the hydrogel more hydrophobic. As a consequence, the water content of the gel was reduced and significant volume shrinkage was observed. The replacement of the hydrophobic fluorescein with hydrophilic derivatized-fluorescein recovered the hydration state of this hydrogel and restored its equilibrium volume. This seems to indicate that the responsiveness of this hydrogel depends on the hydrophobicity of the antigen.

Another antigen-responsive hydrogel has been characterized by (Miyata et al., 1999). A rabbit immunoglobulin G (rabbit IgG) antigen and goat anti-rabbit IgG (GAR IgG) antibody were grafted onto a poly(vinyl)/poly(acrylamide) hydrogel. Interactions between the antigen and antibody formed affinity cross-links in the gel. The presence of free rabbit IgG caused dissociation of these non-covalent links leading to the gel's swelling.

In this paper we report the preparation and characterization of an antigen-responsive hydrogel membrane using dextran as a polymeric network with divinyl sulfone as a cross linking reagent. We demonstrate that this membrane has gating properties for selective diffusion that are responsive to the presence of free antigen.

Materials and Methods

Sheep anti-FITC total IgG antibody was obtained from Micropharm Ltd, UK. Sodium fluorescein was from Fluka; N-hydroxysuccinimide and ethylcarbodiimide from Lancaster Synthesis Ltd, UK. All other chemicals were obtained from Sigma-Aldrich, UK.

Preparation of Antigen-Responsive Hydrogels

Na_2CO_3 (4 g) was added to a solution of 10 g dextran in 150 mL of water, followed by the addition of 1.2 mL of 97% DVS. The reaction was left to proceed for 1.5 h, after which the pH was adjusted to 3.5 using HCl. It was then dialyzed extensively against distilled water and freeze-dried.

The dextran-DVS conjugate (4 g) was dissolved in 60 mL of dimethyl sulfoxide and 20 mg of fluorescein isothiocyanate were added; after 1 h a further 40 mg was added. The mixture was heated at 96°C for 2 h. After cooling, 150 mL of ethanol was slowly added whilst stirring. The solution was then dialyzed against distilled water and freeze-dried.

To determine the FITC ratio in the product, the absorbance of a 0.2 mg/mL of dextran-DVS-FITC was measured. Based on the standard curve of sodium fluorescein, the weight ratio of FITC to dextran was found to be 0.6 mg/g dextran.

To prepare the antibody-dependent hydrogel, 400 mg of dextran-DVS-FITC were dissolved in 2 mL of water followed by 2 mL of sheep anti-FITC IgG antibody solution (50 mg/mL in pH 7.4 phosphate buffer). This resulted in a gel containing an immobilized FITC concentration of 0.14 mM, approximately an order of magnitude higher than the

immobilized IgG concentration. When used for transport experiments, the mixture was poured onto a nylon mesh (0.3 mm thickness) to give the gel more mechanical strength.

Characterization of Binding Interactions

Binding of IgG to free sodium fluorescein, and to conjugated dextran-DVS-FITC was studied using isothermal titration calorimetry (ITC) in an OMEGA ITC (from Microcal Ltd, Northampton, MA). Titrations were run at 20°C and samples were degassed at 15°C prior to use. The protocol involved 30 additions of 5 μl of titrant sample automatically added at 2 min intervals into the IgG solution and stirred at 300 rpm. The heat evolved after each injection was measured by the cell's feedback network as differential heat effects between the sample and its reference cell. Control experiments were carried out using identical titrant injections into a cell-containing buffer without IgG; relative heats of dilution and mixing were subtracted from the heats of binding relative to the real titration experiment. Final experimental results, represented by heat-developed versus cell ligand/IgG ratio, were fitted to a one-binding-site-per-monomer model using Origin ITC Data Analysis Software (Microcal Ltd).

FT-IR Analysis of Antigen-Responsive Hydrogels

Freeze-dried hydrogel samples were prepared and mixed with 95% potassium bromide powder. This was then pressed into tablets under vacuum. Infrared spectroscopy was carried out using a Bruker-equinox 55 FT-IR spectrophotometer. For each sample 60 scans were recorded from 3,200 to 600 cm^{-1} with a resolution of 1 cm^{-1}.

DVS Substitution in Dextran Determined by NMR

Proton NMR spectra were recorded on a Bruker AVANCE 300 spectrometer at 300.13 MHz. Chemical shifts (δ) are reported in ppm using deuterium oxide. The composition of the grafted dextran was estimated from the NMR by comparing the CH groups (δ 6.4 ppm, ^{1}H per double bond in DVS) and the C1-protons of the dextran main chain (δ 4.88 ppm, 2H per unit). Dextran (500,000 kDa) was used for the calculation. (DVS/dextran means the number of DVS in 1 mole.)

SEM Analysis of Antigen-Responsive Hydrogels

The morphology of the gel was studied using a Jeol 6310 SEM equipped with a cryo-stage and energy-dispersive X-ray. The hydrogel was prepared as above and cut in half and placed in either phosphate buffer (pH 7.4) or a similar buffer containing 0.1 mg/mL sodium fluorescein (approximately an order of magnitude higher than the immobilized FITC concentration). A control gel was also prepared that contained BSA, rather than IgG; it was treated the same way. A sample of the hydrogel was rapidly frozen in liquid nitrogen, and then introduced into the SEM chamber, which was pre-cooled to −160°C. At this stage the sample was heated to −80°C to sublime the surface water. The sample was then cooled again to −160°C and gold sputtered for 3 min. The samples were scanned at a magnification of 1,000×.

Confocal Laser Scanning Microscopy

Images were taken at 14 μm intervals through the depth of gel slabs of total thickness 0.7 mm using a Zeiss LSM510 confocal laser scanning microscope. A set of images in the form of a TIF stack was collected for gels in the presence and absence of competitor. Image stacks were analyzed using the public domain IMAGEJ (http://rsb.info.nih.gov/ij/) package in conjunction with the VolumeJ plugin (http://bij.isi.uu.nl/index.htm). All image contrast adjustments were applied equally to each stack.

Exclusion-Based Swelling Measurements

The swelling ratio (i.e., the ratio of the gel volume in the presence of competitor to that in the absence of competitor) of gels was determined by a recently reported method (Noomrio et al., 2005). Briefly, 1 ml mixtures of gel precursors as described above were equally cast in two sides of standard 4.5 mL plastic, 3 mL spectrophotometer cuvettes (path length 1 cm) such that the gel layers did not impinge on the optical path in the cuvette. Blue dextran (6,000 kDa, 0.2 mg/mL) in a pH 5.0 buffer was placed in the cuvette and the absorbance increase was measured as the gel imbibed-fluid and excluded-dye concentrations increased. The experiment was performed in the presence and absence of a solution of 0.1 mg/mL sodium fluorescein. As the total system volume does not change the volume change of the gel can be calculated from a simple balance equation:

$$V_{tot} = V_l + V_g$$

$$D_{tot} = V_l D_l \text{ (as the dye cannot enter the gel phase)}$$

$$\therefore V_g = V_{tot} - \frac{D_{tot}}{D_l}$$

where.∴.

V_{tot} = total volume; V_{l} = liquid volume; V_{g} = gel volume; D_{tot} = total dye concentration; D_{l} = dye concentration in the liquid phase.

Measuring Transport Across Antigen-Responsive Membranes

Transport of blue-dextran molecules (12 kDa) through the hydrogel membrane was measured using a diffusion cell consisting of donor and receptor chambers of equal volumes of 4.4 mL. The surface area for diffusion was 4.6 cm^2. The gel was synthesized as described previously onto a nylon mesh

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to form a membrane. This was then mounted between the chambers and equilibrated with 20 mM acetic acid buffer (pH = 5, I = 0.1 M). The donor chamber was connected to a reservoir of blue-dextran solution through a pump. The receptor chamber was connected to a flow cell mounted in a spectrophotometer (Shimadzu, UV-1601) via a closed-loop recycling system. Diffusion of blue-dextran across the membrane was continuously monitored by detecting absorbance changes in the flow cell at 610 nm.

Results and Discussion

The synthetic chemistry is described in Figure 1. The use of dextran as the backbone of the antigen-responsive polymer and DVS chemistry as the basis for the covalent links was based on the following considerations. Dextran has low toxicity and is easily degraded by dextranase enzymes, which are found in the large intestine. Therefore, dextran hydrogels have promise for use in drug delivery systems or in the controlled release of bioactive agents. Dextran hydrogels cross-linked with other polysaccharides have also been developed by other groups (Kim et al., 1999; Van Dijk-Wolthuis et al., 1997). Recently, a series of hydrogels was fabricated from CM-dextran in our laboratories using carbodiimide chemistry (Zhang, 2005; Zhang et al., 2005). This approach is quicker and easier than the triazine method that we previously employed (Mayes et al., 1990; Tang et al., 2003, 2004), and allows greater control over charge density and the degree of cross-linking in the gel. Thus the balance between degradation rates and mechanical properties is very easily controlled. However, when this approach was applied to the grafting of proteins to a polymeric network, inter cross-linking between proteins occurred. Another drawback of the carbodiimide approach is that the chemical conjugation of the antibody in the modification reaction usually occurs at the ε-amino group of lysine. This reaction is non-specific, and the presence of multiple reactive groups results in a protein or antibody with uncontrollable modification degrees. The random modification of protein or antibody often causes the loss of binding activity and probably, the low sensitivity of such hydrogels to antigens.

DVS is a homobifunctional reagent. Its vinyl groups can participate as electrophiles in the reactions of nucleophilic addition (Friedmann et al., 1965). Free amino groups of lys residues are the obvious targets of DVS conjugation with proteins. However, where thiol groups are present, the conjugated double bond of the vinyl sulfone reacts with these preferentially, as shown in several previous studies (Elbert et al., 2001; Friedmann et al., 1965; Morpurgo et al., 1996). In the current study, protein conjugation to the polymer was carried out at pH 7.4 at which lysine amino groups will be highly protonated and thus unreactive as a nucleophile. For these reasons, we believe that the linkage joining the antibody to the DVS-dextran will be an S–C bond.

Dextran was first grafted with a DVS group. It was noticed that as reaction time increases more DVS groups were found

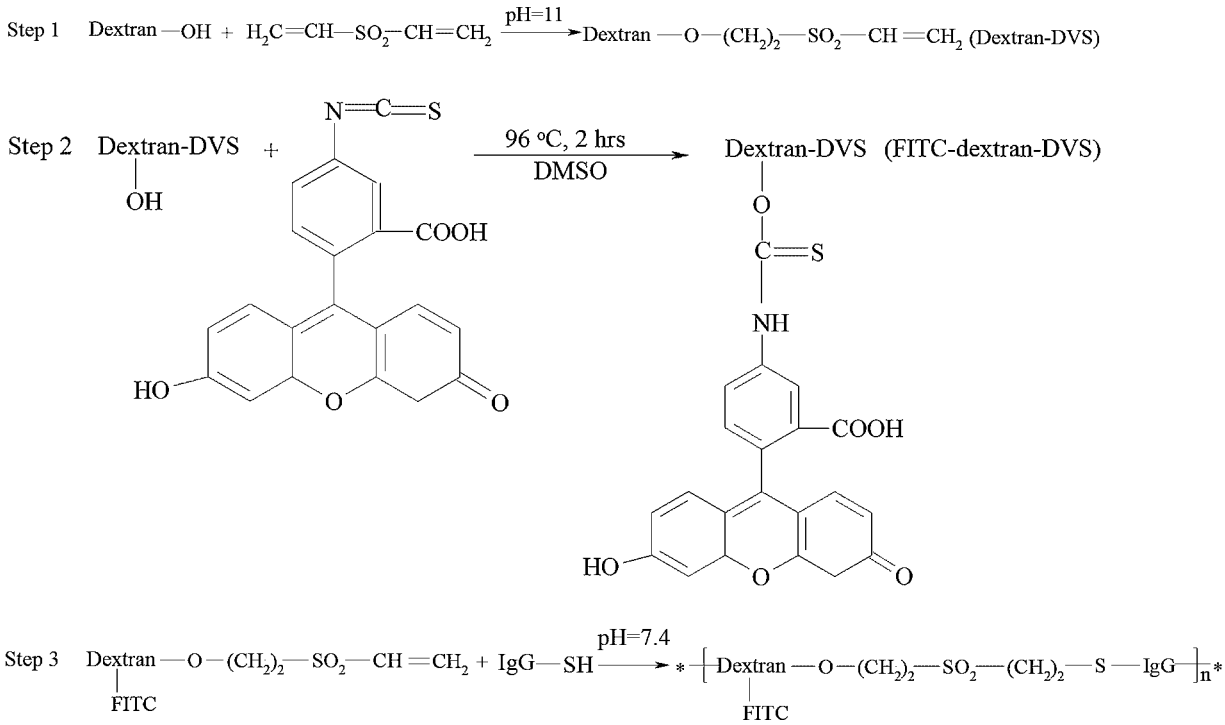

Figure 1. The chemistry of the FITC-dextran-DVS-antibody hydrogel preparation.

in the dextran chains. If the reaction lasted too long, the dextran solution became so viscous that the freeze-dried residue became very difficult to re-dissolve in distilled water in the subsequent gelation reaction, possibly because the DVS-dextran could interlink with the hydroxyl groups in the dextran, thus enhancing the polymer chain's expansion. The optimum reaction time was 1 h under the reaction condition as described above. A control hydrogel was made with bovine serum albumin (BSA) replacing the antibody.

The gel was characterized by ^{1}H NMR and Fourier Transform Infrared (FTIR) spectrometry. NMR spectra are shown in Figure 2. The composition of the grafted dextran was estimated as 200 DVS/dextran chain from the NMR, by comparing the CH groups (δ 6.4 ppm, ^{1}H per double bond in DVS) and the C1-protons of the dextran main chain (δ 4.88 ppm, ^{2}H per unit) where a dextran molar weight of 500 kDa was used in the calculation.

That FITC was successfully incorporated into the dextran-DVS is demonstrated by the peak at 1,156 cm^{-1} in the FTIR spectrum shown in Figure 3. The antibody was then successfully grafted onto the FITC-dextran-DVS, which is shown by the NH_2 peak at 1,550 cm^{-1}. Absorbance measurements at 490 nm in the UV–visible spectrum also confirmed that the FITC was successfully incorporated into the hydrogel.

Binding of FITC-dextran-DVS and of fluorescein to the antibody was compared in pH 5.0 buffers using ITC. It is known that the antibody–antigen interaction is pH dependent, and becomes weak at low pH. Acetate buffer of 20 mM and pH 5.0 was chosen because of the low ionic strength of acetate solution and the moderate antigen–antibody binding strength at relatively low pH. The antigen exchange reaction took place readily at pH 5. ITC has found extensive utility in ligand binding studies. The ligand is titrated into a solution containing the binding protein (here IgG), and the heat evolved or absorbed during binding is measured. Deconvolution of the data obtained from a sequence of additions yields the binding constant, enthalpy and entropy of binding, and the stoichiometry of the interaction. A sheep anti-FITC IgG antibody solution in pH 5 acetic acid buffer was titrated with sodium fluorescein and FITC-dextran-DVS, respectively. Figure 4 (panel 1) shows the raw calorimetric data for the titration of native IgG with Fluorescein, demonstrating the heat evolved (negative exothermic peaks). Panel 2 shows the plot of heat evolved per injection as a function of the molar ratio of IgG to the fluorescein. The smooth solid line represents the best fit to the experimental data using a single-binding-site model. The binding constants of different IgG to fluorescein and FITC-dextran-DVS conjugates are shown in Figure 4. Comparison of the binding constants obtained for free fluorescein and

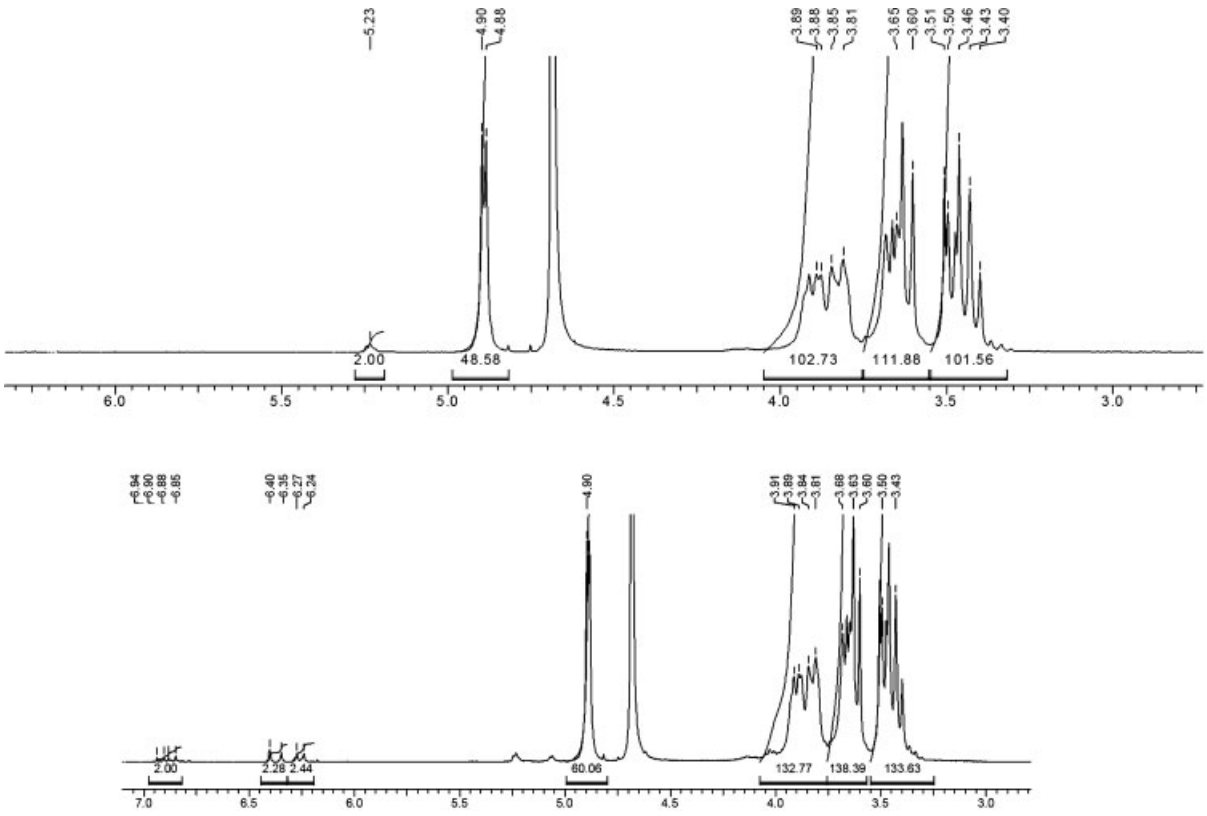

Figure 2. ^{1}H NMR spectra of dextran-DVS.

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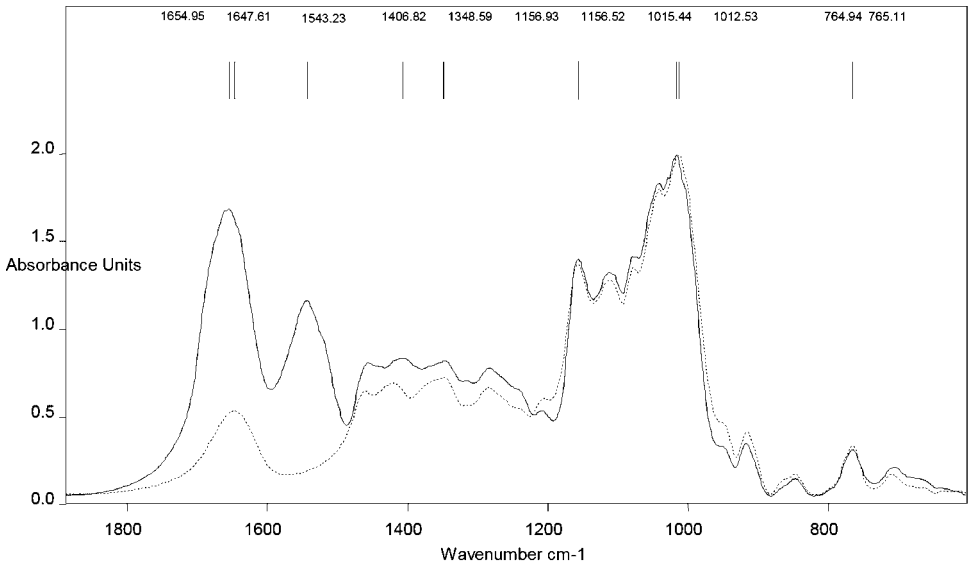

Figure 3. FTIR spectra of the FITC-Dextran-DVS-antibody hydrogel.

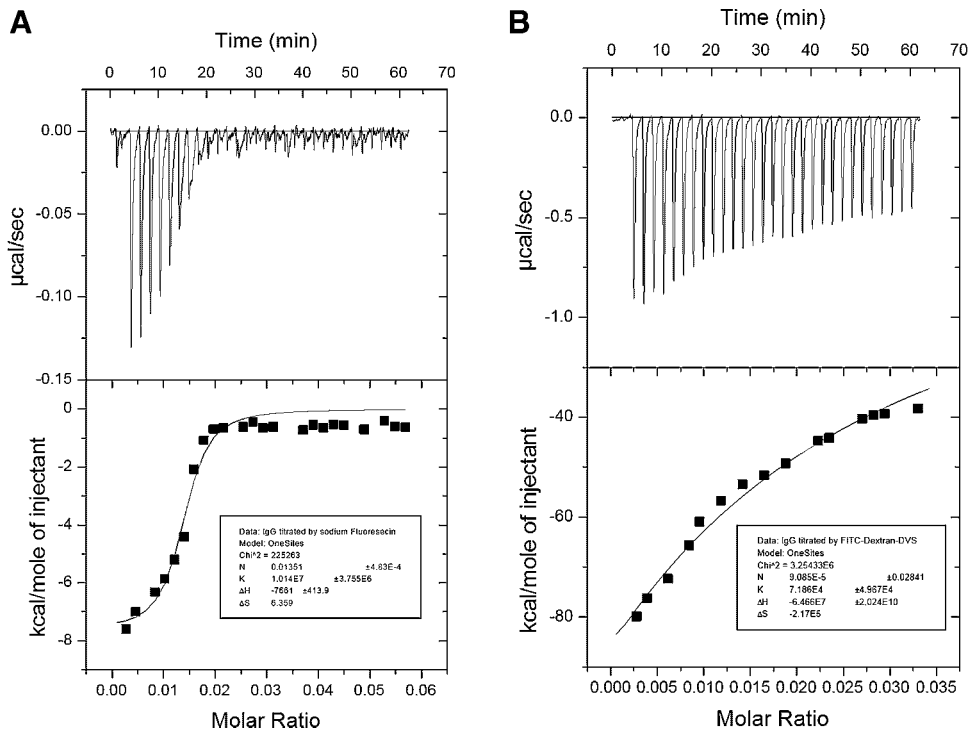

Figure 4. ITC titration curves of IgG titrated with (**A**) sodium fluorescein and (**B**) fluorescein conjugated to dextran-DVS. A: Titration of IgG (5mg/mL) with sodium fluorescein (0.05 mg/mL). B: Titration of IgG (3 mg/mL) with fluorescein conjugated to dextran-DVS (0.03 mg/mL). Titrations were carried out in 20 mM acetate buffer, pH 5.0 containing 0.02% NaN_3. The parameters shown are calculated from fits to a one-binding-site model, (N, Stoichiometry; K, Association constant (M^{-1}); H, Enthalpy (cal mol^{-1}); S, Entropy (cal mol^{-1} K^{-1})).

FITC-dextran-DVS conjugates with IgG show a dramatic reduction in the association constant from 10^7 to 10^4 M^{-1}, indicating a reduced affinity. This is beneficial for the development of antigen-based responsive hydrogels as it means that the external sodium fluorescein will effectively compete with the antigen–antibody interaction and allow the gel to respond. The results also show a much greater energy change in the case of the antibody-dextran conjugate possibly relating from the occurrence of non-specific binding between antigen and the dextran backbone. The negative value observed for the binding entropy for free antibody is probably an artifact of arising from the relative magnitude of experimental error to the limited energy changes observed.

Scanning electron microscopy shows that the structure of a hydrogel incorporating both antibody and antigen undergoes a significant change in response to presence of the free antigen (fluorescein) (Fig. 5). A similar effect is not seen with control gels containing BSA as an inert protein: in this case the structure of the gel, both in the presence and absence of antigen, resembles that of the antibody gel in the presence of antigen. This possibly reflects the higher crosslink density obtained where affinity cross-links augment the covalent cross-links formed during gel synthesis. When free antigen is added these are displaced and the total cross-link density will be similar to that observed for the control gel. There is also the possibility of slight shrinkage of the gels in fluorescein solution at pH 5.0 (panel 5D) resulting from the change of the microenvironment of the hydrogel after fluorescein adsorption. At pH 5.0 in an acetate buffer, fluorescein is protonated and relatively hydrophobic. The non-specific adsorption of relatively large amounts of the hydrophobic fluorescein would render the hydrogel more hydrophobic leading to significant volume shrinkage observed by (Lu et al., 2003).

The SEM images all show structures containing voids with diameters in the micron scale consistent with images we have obtained from other hydrogel preparations (Zhang et al., 2005). We propose that molecular transport through the gels is controlled by changes in the mesh size of the gel walls to these voids and that these changes are reflected in the gross morphological changes observed.

The need to freeze, dry, and coat samples prior to SEM imaging introduces the possibility that the changes seen may result from sample preparation artifacts. To examine this possibility, images of gels were obtained using a confocal laser-scanning microscope. Three-dimensional images constructed from a stack of depth slices through gels in the presence and absence of competitor are shown in Figure 6. These confirm the SEM findings of a competitor induced structural change, in this case observed through the depth as well as the surface of the slab imaged. As the samples remained in a hydrated state throughout, this confirms that the differences observed in the SEM images are not solely attributable to artifacts arising from sample preparation.

The swelling ratio of the antibody gel was determined by a size-exclusion method (Noomrio et al., 2005). The tracer used was a commercial blue dextran preparation with a

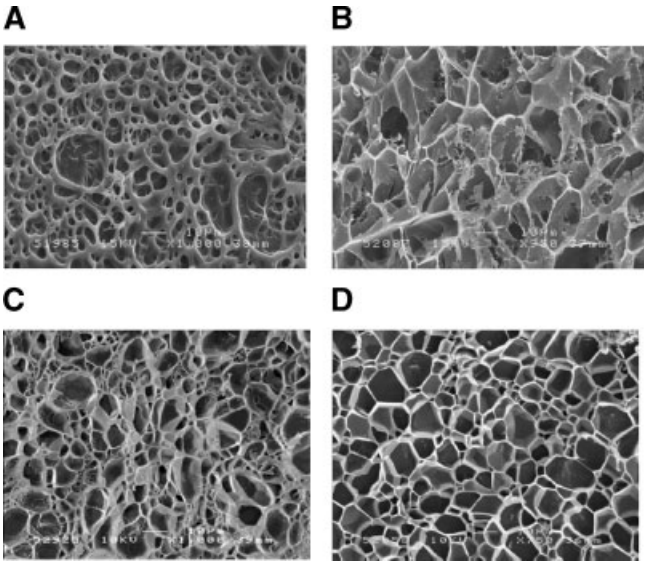

Figure 5. SEM showing the three-dimensional structure of the antigen-responsive hydrogel in (**A**) pH 7.4 buffer and (**B**) pH 7.4 buffer containing 0.1 mg/mL sodium fluorescein; and a control gel (containing BSA in place of the antibody) in (**C**) pH 7.4 buffer and (**D**) pH 7.4 buffer containing 0.1 mg/mL sodium fluorescein.

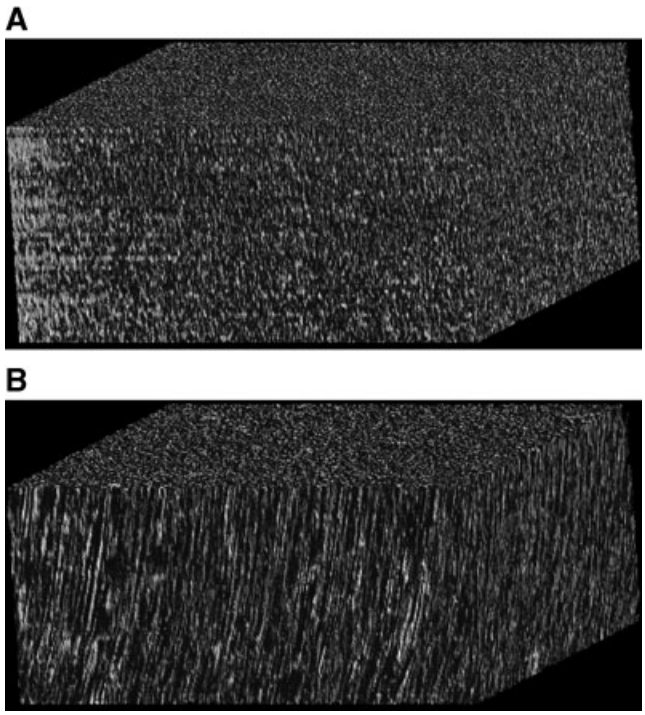

Figure 6. Confocal laser scanning microscopy images of gel in the absence (**A**) and presence (**B**) of competitor. The images shown are of a square gel section dimensions 1.4 × 1.4 mm.

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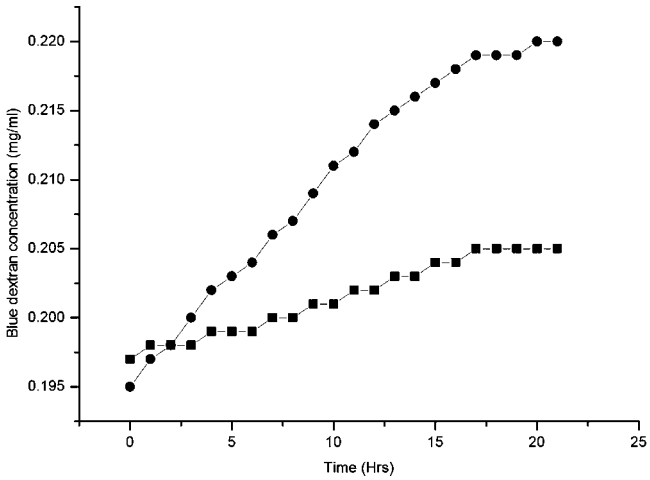

Figure 7. Diffusion of Blue dextran (6,000 kDa, 0.2 mg/mL) into a hydrogel slab in the presence (▲) and absence (■) of 0.1 mg/mL sodium fluorescein at pH 5.0.

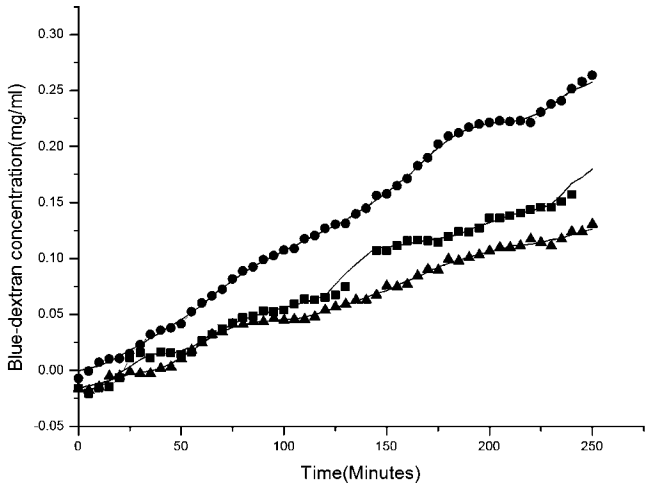

Figure 8. The reversibility of blue dextran (4 mg/mL of a 12 kDa preparation) transport through a hydrogel membrane in response to the addition and removal of sodium fluorescein (0.1 mg/mL) at pH 5.0. ▲ Blue dextran – virgin membrane; ● Blue dextran + sodium fluorescein; ■ Blue dextran after washing to remove sodium fluorescein.

quoted molecular weight of 6,000 kDa. Concentration changes were determined from increases in optical absorbance in the liquid phase (610 nm) using a standard laboratory spectrophotometer. Swelling ratios were determined from a simple mass balance of the system assuming complete exclusion. Figure 7 shows the increase in concentration of blue-dextran as a function of time for the antigen-sensitive gel in the presence of 0.1 mg/mL sodium fluorescein. Equilibrium was approached after 16 h. The swelling ratio in free fluorescein solution was 2.84 compared with 0.13 in the absence of free fluorescein. This confirms that the gel is responding to free fluorescein. Gels produced using BSA instead of antibody did not show a similar change in swelling ratio indicating a specific effect.

For the transport permeability experiments, an antigen-responsive hydrogel was prepared as described previously and poured onto a nylon mesh. The mesh was placed into a diffusion cell. The membrane was washed with pH 5 buffer and the donor chamber was filled with 4 mg/mL of blue dextran (12 kDa). Diffusion of the blue dextran through the membrane was measured by detecting changes in the absorbance at 610 nm over time. The blue dextran was then washed out and blue dextran solution containing 0.1 mg/ml sodium fluorescein was pumped into the donor chamber. The receptor chamber was filled with 0.1 mg/mL sodium fluorescein and again diffusion across the membrane was detected by measuring changes in receptor-chamber absorbance at 610 nm. To determine if gel swelling was reversible, the chambers were then washed out and blue dextran solution was added again to the donor chamber and pH 5 buffer to the receptor chamber. The absorbance was compared to a blue dextran standard curve to determine the concentration of blue dextran that moved across the membrane. In the absence of free antigen (i.e., sodium fluorescein), significantly less blue dextran moved across the membrane into the receptor chamber. As shown in Figure 8, in the presence of 0.1 mg/mL sodium fluorescein the hydrogel opens and allows blue dextran through more rapidly. The receptor chamber had to be filled with sodium fluorescein as the sodium fluorescein changes the color of the blue dextran solution. All solutions containing sodium fluorescein were then removed from the system and the concentration of blue dextran in the receptor chamber was reduced to approximately basal level. Although this does not represent an exhaustive removal of antigen, as the membrane gel volume is $<1\%$ of the system volume, any residual fluorescein will be vastly diluted. It is likely that some bound material will remain and this is reflected in the fact that while the transport rate for the washed membrane is lower than that observed in the presence of fluorescein solution it is higher than observed for the virgin membrane.

The results described above provide a demonstration that hydrogel membranes with integral antibody–antigen interactions incorporated into their structure can be fabricated. This has been demonstrated using an antigen/antibody couple based on fluorescein as the hapten. It is also clear from these experiments that these antigen-responsive hydrogel membranes respond effectively to the presence of free antigen in a manner that alters their permeability. Thus, transport of large solutes through these membranes was selectively responsive to the presence of free fluorescein in the external medium. Since antibodies can be raised to a vast range of haptens, this demonstration provides in principle the basis for the development of membrane hydrogels responsive to an equally extensive variety of

molecular species. Such membranes may have potential for a wide scope of applications (e.g., drug delivery and biosensors) in the biomedical sciences.

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