Temperature Responsive Pore-Filled Membranes Based on a BSA/Poly(*N*-isopropylacrylamide) Hydrogel

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ABSTRACT: A temperature-sensitive hydrogel based on a copolymer of BSA and poly(*N*-isopropylacrylamide) (PNIPAAm) has been synthesized using carbodiimide chemistry. Fourier transform infrared spectroscopy confirmed primary complex formation between carbodiimide-activated carboxylic acids on the protein with protein amino groups. As a result of temperature-induced

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conformational changes in PNIPAAm grafted onto the protein backbone, these protein hydrogels show significant morphological changes in response to temperature. The structural changes of the gels in response to temperature were assessed using scanning electron microscopy, and the effect of temperature on their balance of hydrophobicity was found using turbidity measurements. Composite pore-filled membranes formed by impregnating glass fiber filters with the polymer mixture prior to gelation were used to determine permeability changes in response to temperature using both low (riboflavin) and intermediate (lysozyme) molecular weight diffusates. Clear correlation was found between changes in morphology, turbidity, and gel permeability as the gel temperature was increased from 24–37°C. In the case of permeability studies, significant transport of lysozyme only occurred at temperatures above the lower transition temperature of the hydrogel, suggesting the gel was acting as a mechanical "valve" to control flux. Adv Polym Techn 27: 27–34, 2008; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/adv.20113

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Introduction

Responsive polymers and hydrogels based on a number of mechanisms have been studied extensively for a wide range of applications.[1] One of the most widely studied systems is based on poly(*N*-isopropylacrylamide) (PNIPAAm) gels, which exhibit a temperature-induced volume phase transition in water upon heating to above 32°C.[2] PNIPAAm gels are the most commonly studied temperature-sensitive materials mainly because of their sharp phase transition, the proximity of their transition temperature (around 32°C), to mammalian physiological temperatures, and the ease with which the transition temperature can be adjusted by copolymerization with other molecules.[3–7] As the temperature is increased through the transition temperature value, these gels undergo a temperature-induced collapse from an extended coil to a globular structure. This transition is revealed on the macroscopic scale by a sudden decrease in the degree of swelling of PNIPAAm gels.[8]

Conjugates of PNIPAAm and biological macromolecules have significant potential for application in medicine and biotechnology, particular for drug delivery, enzyme and cell immobilization, and in an affinity-precipitation-based immunoassays.[9–12] A common method for synthesizing PNIPPAm biological macromolecules conjugates is based on water-soluble carbodiimide reactions between activated functional groups of macromolecules.[13–16] We have previously used carbodiimide chemistry to produce responsive hydrogels based on cross-linked carboxymethyl dextran (CM-dextran) both with and without coupled receptor proteins.[17]

Preliminary studies with gels based on PNIPAAm alone resulted in materials that were mechanically weak and resulted in structural damage when exposed to a number of temperature cycles. This resulted in loss of PNIPAAm into solution. To overcome this problem, we have used a protein to improve the stability of the gels produced.[18] In the study reported here, a PNIPAAm hydrogel based on bovine serum albumin (BSA) as a model protein backbone was prepared using carbodiimide chemistry. High concentrations of BSA were used to promote hydrogel formation through the anticipated formation of ester bonds between the amino acid and carboxyl groups in the BSA and also amide bonds from PNIPAAm—COOH with BSA—NH_2.[15] To test this hypothesis, the hydrogels produced were characterized by Fourier transform infrared spectroscopy (FT-IR).

The structural properties of the gels were investigated using scanning electron microscopy (SEM) with cryofixation and cryofracturing techniques and spectrophotometric phase transition studies. Finally, the transport characteristics of temperature-responsive pore-filled membranes based on these gels were characterized in diffusion experiments.

Materials and Methods

MATERIALS

BSA and lysozyme were obtained from Sigma–Aldrich (UK). All other chemicals were of reagent grade and obtained from Lancaster Synthesis Ltd. (UK).

HYDROGEL SYNTHESIS

N-Isopropylacrylamide (NIPAAm) was purified by recrystallization from *n*-hexane. Poly(NIPAAm) with one terminal COOH group was synthesized by free-radical polymerization, using an approach based on published protocols.[19,20] The dried polymer was purified by precipitation in hot deionized water (>40°C) and washed at this temperature prior to dissolving in deionized water at 25°C. The final polymer preparation was recovered from the solution by freeze-drying.

Thermally sensitive hydrogels based on poly(NIPAAm) terminated with a carboxyl group were prepared by chain transfer polymerization, using 3-mercaptopropionic acid (MPA) as a chain transfer agent, as described by Chen and Hoffman.[20] The active-ester PNIPAAm was prepared by reaction of the carboxyl terminal group with *N*-hydroxysuccinimide (NHS) using 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) as an activating reagent in deionized water at room temperature. According to the expected cross-linking mechanism shown in Fig. 1, the activated ester bond in PNIPPAm should react with the amino acid group on the BSA. Also it is likely that additional links will be formed between external carboxylic and amino groups on the BSA molecule.

Hydrogels were prepared as follows: 200 mg of PNIPAAm-COOH was dissolved using 2 mL distilled water with stirring and the resulting solution degassed. Two hundred milligrams of EDC and 40 mg of NHS dissolved in 1 mL deionized water was added to the PNIPAAmCOOH solution and stirred for 10 min. Then 400 mg BSA dissolved in 2 mL distilled water was poured into the reaction solution with continuous stirring for another 10 min at which point the mixture was cast and allowed to gel as required for subsequent analysis.

EDC is used to activate the COOH groups in PNIPAAm, which then react with NHS to form a relative stable ester, which further reacts with amino groups on the BSA. A large excess of EDC is used as some will be lost to competing reactions and some will be consumed in the formation of internal cross-links between COOH and NH_2 in the BSA molecules. Gels were exhaustively washed until no more leachable PNIPAAm was observed, prior to their use in transport experiment or SEM analysis.

Infrared spectroscopy was performed on a Bruker-Equinox 55 FT-IR spectrometer. Freeze-dried gel 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}.

HYDROGEL MORPHOLOGY

The morphology of the hydrogel at different temperatures was examined using a Jeol 6310 SEM equipped with a cryostage and energy-dispersive X-ray (EDX). The hydrogel was incubated at 25 and 37°C with a Tris buffer before a sample of the hydrogel was rapidly frozen in liquid nitrogen then introduced into the SEM-chamber precooled to a temperature of ca. −160°C. The stage was the heated

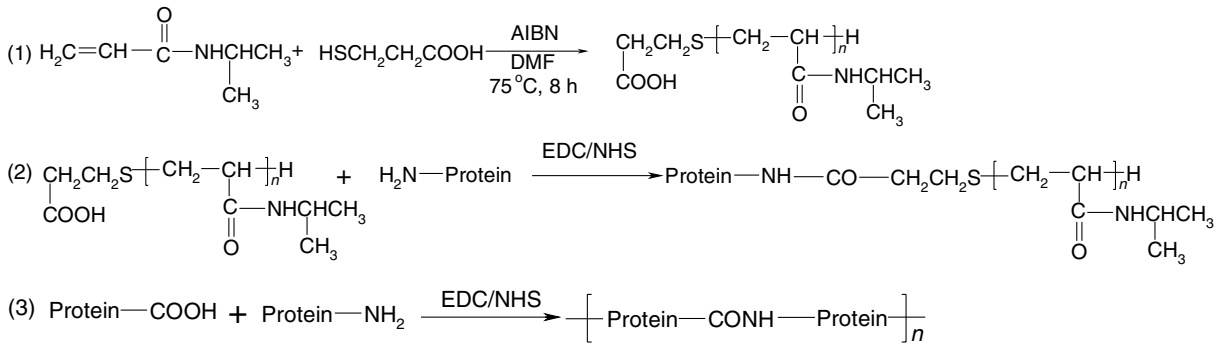

FIGURE 1. Synthesis route for the preparation of BSA/PNIPAAm hydrogels.

to a temperature of ca. −80°C to sublimate the surface water. After cooling to −160°C, the sample was gold sputtered for 3 min. The sample was scanned at a magnification of 2000×. Differences in structure were assessed using the ImageJ image analysis software (http://rsb.info.nih.gov/ij/).

PHASE TRANSITION STUDIES

The phase transition behavior of the synthesized hydrogels was studied by determining the optical transmittance of the system as a function of temperature; studies were conducted at 600 nm over the temperature range 20–40°C, using a UV–visible spectrophotometer (Shimadzu 1601) with a hydrogel slab mounted in a 5-mm glass cuvette.

MEMBRANE TRANSPORT EXPERIMENTS

Hydrogel composite membrane disks were prepared by cross-linking the BSA/poly(NIPAAm) within the pores of sintered glass filter disks (2.7μm Millipore). Dry sintered glass filter disks were submerged into the polymerization mixture. The disks were left in the polymerization mixture for 6 h. The disks were removed from the hydrogel that had formed and were exhaustively washed in deionized water.

The transmembrane transport of protein was investigated using riboflavin and lysozyme as the test molecules. Experiments were conducted in a diffusion cell consisting of donor and receptor chambers of equal volumes of 4.4 mL.[21] 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 test molecule reservoir via a pump. The receptor chamber was connected to a UV–visible spectrophotometer (Shimadzu 1601), to allow test molecule diffusion across the membrane to be monitored and logged from optical density changes (riboflavin at 440 nm and lysozyme at 280 nm). The effect of temperature was investigated by varying the temperature of the water bath containing the diffusion cell and solute reservoir.

Results and Discussion

Figure 2 shows the FT-IR spectra obtained for gels based on cross-linked BSA alone and BSA/PNIPAAm hydrogels. There is one distinct absorption peak at 3310 cm^{-1} from the protein spectra,

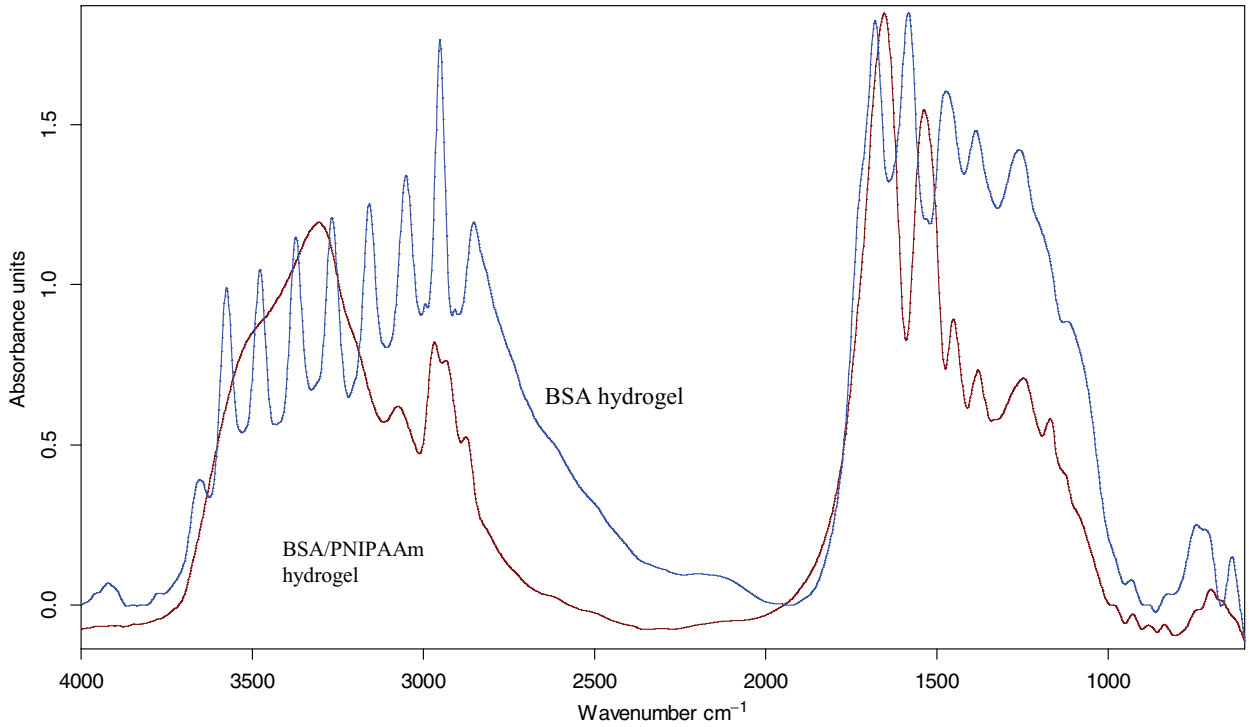

FIGURE 2. FT-IR spectra of BSA and BSA/PNIPAAm hydrogels.

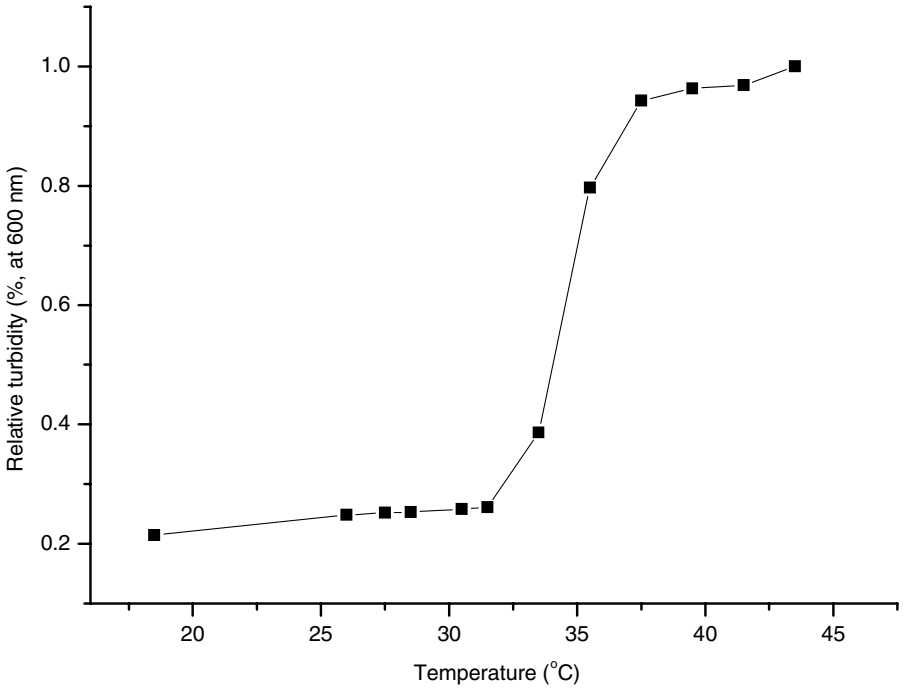

FIGURE 3. Effect of temperature (20–40°C) on the absorbance of a BSA/PNIPAAm hydrogel slab at 600 nm in 20 mM Tris buffer (pH 7.4).

which can be attributed to stretching of hydrogen bonds with NH groups. Other peaks characteristic of the NIPAAm structure are the antisymmetric stretching vibration of the CH_3 group at 2927 and 2876 cm^{-1} and a strong vibration peak for the C=O groups at 1651 cm^{-1}. The mixture vibration of CN and NH appears at 1378 cm^{-1}. Even though the amide signal from BSA could cover the linkage bond between PNIPAAm and BSA, the significant difference between the gels confirm the successful gelation occurs as expected. Characteristic peaks of the NIPAAm structure are the antisymmetric stretching vibration of the CH_3 group at 2927 and 2876 cm^{-1} and a strong vibration peak for the C=O groups at 1651 cm^{-1} The distinct absorption peak at 3310 cm^{-1} is from the protein spectra, which can be attributed to stretching of hydrogen bonds with NH groups.

The transition temperature was determined by measuring absorbance at 600 nm for a slab of hydrogel as temperature was increased from 20 to 40°C (Fig. 3). The gel remained clear below 32°C and became totally opaque above 37°C. These changes were fully reversible with temperature and provide further evidence that PNIPAAm was successfully grafted into the hydrogel network. Once the transition temperature was reached, individual PNIPAAm chains collapsed prior to aggregation. This lead to increasing scattering of light in the solution, resulting

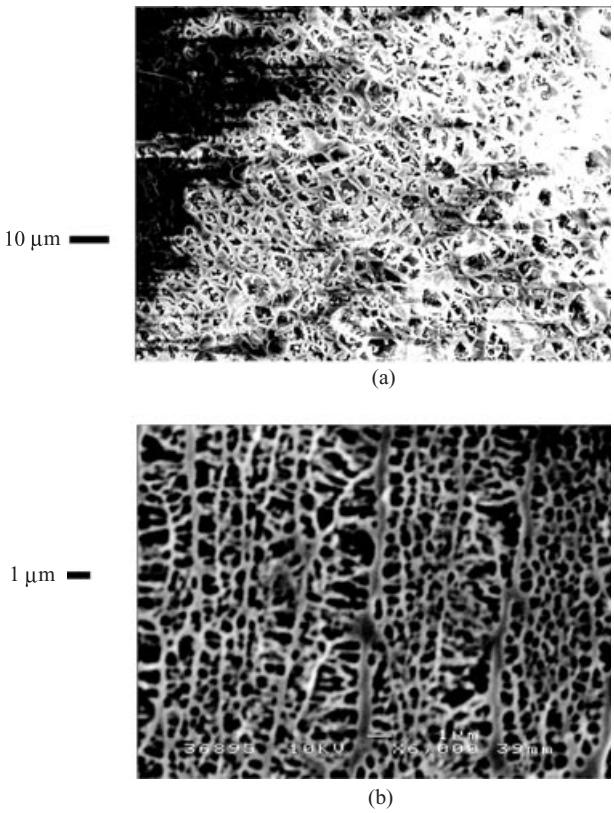

FIGURE 4. Scanning electron micrographs of BSA/PNIPAAm hydrogel (a) at 25°C and (b) at 37°C.

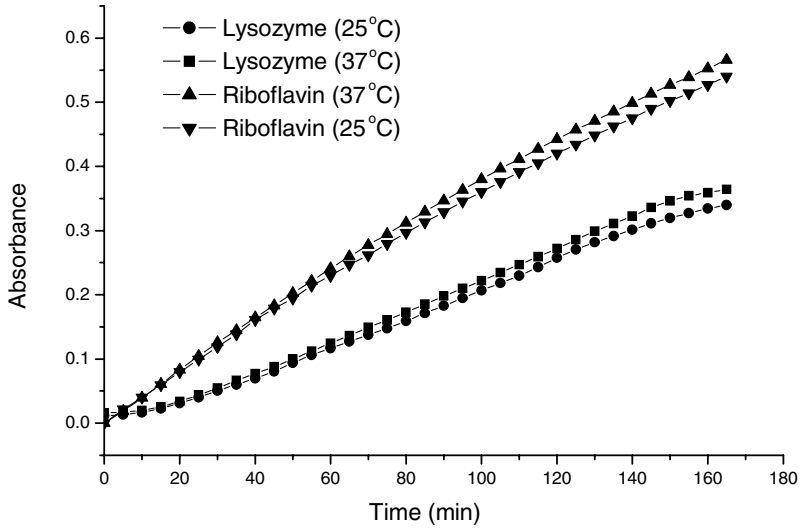

FIGURE 5. Permeation of riboflavin and lysozyme across an unmodified sintered glass filter disk at 25 and 37°C.

in increased turbidity. Further increases in temperature led to the appearance of two phases: one composed of collapsed gel that had expelled most of its associated water and the other the water itself.[22] Thus, the gel underwent a phase transition from a hydrophilic to a hydrophobic structure.

The structure of the BSA/PNIPPAm hydrogel was observed using SEM. Figure 4 shows a characteristic voided structures similar to that reported by Zhang et al.[17] for a pH sensitive hydrogel. Comparison of the void sizes determined by image analysis confirms that the porosity the gel changes from an expanded hydrated state below the transition temperature (Fig. 4a, void mean area 9 μm^2), while above the transition temperature dehydration leads to a more compact structure (Fig. 4b, void mean area 0.8 μm^2).

Gel permeability was quantified using composite membranes with gel synthesized in situ within the pores of a sintered glass filter to provide a robust membrane for use in transport studies. Below the transition temperature hydrogel within the pores was swollen as shown in Fig. 4, acting as a barrier to molecular transport. Above the transition temperature hydrogel collapse leads to incomplete pore blockage enhancing the transport of test molecules.

Figures 5 and 6 show results obtained for the transmembrane transport of riboflavin (at 440 nm)

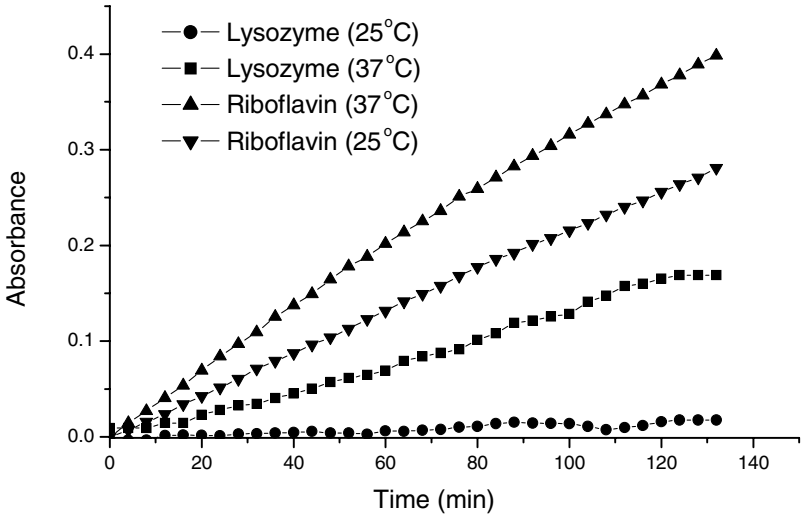

FIGURE 6. Permeation of riboflavin and lysozyme through a BSA/PNIPAAm pore-filled composite membrane.

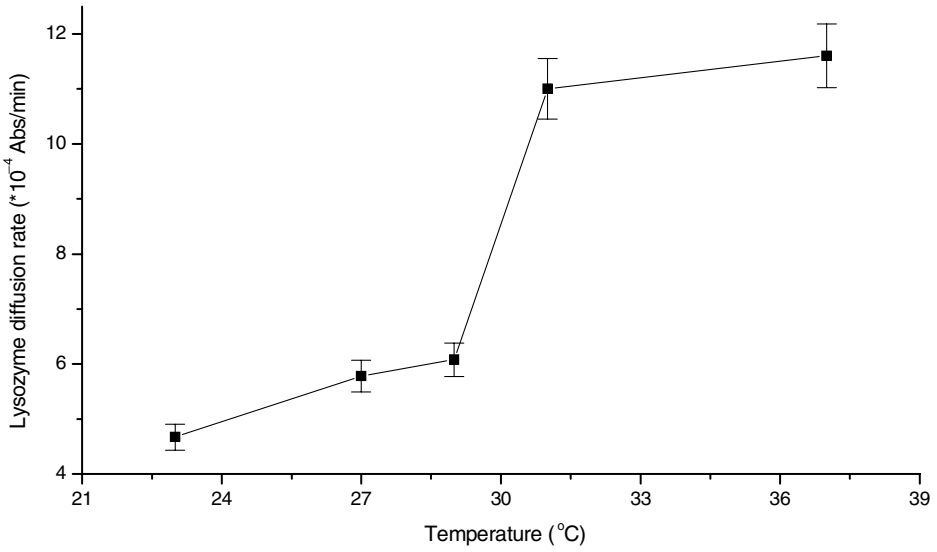

FIGURE 7. Effect of temperature on the permeation of lysozyme through BSA/PNIPAAm gel composite membranes.

and lysozyme (at 280 nm) at 25°C and 35°C through the pore-filled hydrogel membrane compared with a control unfilled glass filter membrane. Transport through the blank glass filter membrane showed no effect of temperature on transmembrane flux, although the smaller riboflavin molecule shows a higher flux than lysozyme (Fig. 5). In the case of the pore-filled membrane, lysozyme was completely rejected at 25°C, suggesting that in the swollen state the hydrogel both completely rejects lysozyme and completely occludes the pores. At 37°C, lysozyme transport was almost as high as for the bank filter. Similarly, riboflavin transport was faster at 37°C than that at 25°C mirroring changes in swelling (Fig. 6).

Lysozyme transport in a gel-substituted membrane was measured over a range of temperatures with the results shown in Fig. 7. These show that, as expected, the effect of temperature on transport mirrors the temperature-dependant turbidity changes shown in Fig. 3, highlighting the role of the PNIPAAm phase change in the control of molecular transport in response to changes in external temperature.

Conclusions

This study demonstrates a facile synthesis protocol for the formation of a temperature-sensitive polymer. SEM and turbidimetric studies confirm the occurrence of temperature-dependent conformational changes and indicate that these are accompanied by significant changes in swelling. Although some of the structural features seen in SEM images may result from sample preparation artifacts, images obtained from confocal laser scanning microscopy show similar morphological features in native hydrogels. Transport studies with the gel loaded into the pores of a diffusive filter show that it can provide the basis for the formation of responsive membranes, which show temperature-dependent changes in permeability. It is difficult to correlate the gross morphological changes in the gel with changes in permeability as the voids are considerably greater in size than the tracer molecules used in permeability measurements. This suggests that the properties of the void walls will be the critical factor in controlling transport through the gel phase.

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