Temperature-Sensitive Hydrogels: A Gentle Way of Concentrating Cellulase Enzymes

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Summary: The swelling behaviour of a poly(N-isopropylacrylamide) temperature sensitive hydrogel in fermentation broth was determined as a function of the temperature and compared with that in water. The feasibility of the gel as a device for concentration of cellulase enzymes, produced by the fermentation of the fungus *Trichoderma reesei* RUT C-30, was also studied. For the same particle size, gels with different composition showed differences in the swelling time and in the extent of swelling. The time required for collapsing was independent of both the monomer concentration and the degree of crosslinking. During swelling, the gels absorb water and low molecular weight solutes (<100 g/mol), excluding macromolecules such as proteins (>10 kD). The enzymes were concentrated in the solution non- absorbed by the gels. Mass balances for fermented broth, total soluble protein and enzyme activity showed a good recovery.

Keywords: hydrogels; networks; polyamides; stimuli-sensitive polymers; swelling

Introduction

Traditional methods for enzyme concentration[1–3] are either energy demanding (i.e. ultrafiltration) or harmful to the enzyme (i.e. solvent precipitation). The use of temperature-sensitive hydrogels has been proposed as an alternative method for enzyme concentration.[4–6] This process is useful to separate bioproducts, due mostly to the mild separation conditions and to the reusability of the gel.

The purification method using temperature sensitive hydrogels takes advantage of the change in the gel volume during a phase transition, and is based on the size exclusion of macromolecules. The size exclusion of solutes, the change in volume and the reversibility of the phase transition of the gel can be used for the concentration of solutions containing macromolecules.

The phase transition of poly(N-isopropylacrylamide) hydrogels (pNIPA-HG) occurs at temperatures as low as 31–33 °C. The phase transition is macroscopically visible as a change in volume of the gel particles. As the gel is warmed, it goes from a swollen to a collapsed state. The volume change is reversible upon lowering the temperature.[7] The pNIPA-HG have been widely used in the biotechnology field,[8] i.e. for soy protein isolation,[9] for drug delivery,[10,11] for enzyme concentration[12] and for enzyme immobilization by Polyacrylamide Gel Electrophoresis (PAGE).[13] These hydrogels separate solutes based on the solute molecular weight and the gel composition. Solutes with molecular weights of 10,000 daltons[14] or sizes of 3 nm[15] have been proposed as the low limit for effective separation. The limit can be changed by altering the gel composition. Gels formed by higher monomer concentration or higher crosslinking degree will exclude smaller molecular weight solutes due to smaller cage sizes.

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Experimental Part

Gel Preparation

PNIPA-HG were produced by a free radical polymerization at 4 °C, as detailed elsewhere.[7,9,12] Three levels of crosslinker were used to produce hydrogels with different crosslinking densities referred as low, medium and high; samples are coded as L-HG, M-HG and H-HG.

Swelling Measurements

1.0 g of xerogel (dry basis) was placed in a graduated cylinder; hydrogel volume was determined directly in the graduated cylinder. The volumes of hydrogels with various compositions were measured as the temperature was changed from 4 °C to 45 °C. The gel was immersed in 55 mL of fermented broth. Water was also used as a comparison.

Enzyme Concentration

The enzyme solution of 1.8 filter paper units/mL (FPU/mL)[12] and pH 6.3 was prepared using commercial cellulase enzyme from *Trichoderma reesei* ATCC 26921 (Sigma). 1.0 g of xerogel and a volume of cellulase enzyme solution were retained in a Plexiglas cylinder. The gel volume was measured prior to the raffinate and permeate recovery. The raffinate and the permeate were collected in graduated cylinders and their volumes collected were then measured.

Results and Discussion

Isothermal Equilibrium Swelling

Usually, upon cooling these pNIPA-HG go from collapsed to swollen states; the collapse temperature is about 31 °C.[12] The relative swelling for these hydrogels is defined as the ratio of the hydrogel volume at any temperature to the volume it shows at the collapsed state. This ratio gives information about the increment in volume that the material undergoes at a specific temperature. Figure 1 shows the relative swelling (V/Vmin) of pNIPA-HG in fermentation broth as a function of temperature for several crosslinking densities: low (L), medium (M) and high (H). The relative swelling for these hydrogels is defined as the ratio of the hydrogel volume at any temperature (V) to the volume it shows at the collapsed state (Vmin). It can be also calculated as the ratio of the hydrogel mass at any temperature to the xerogel (dry hydrogel) mass.[15] This ratio gives information about the increment in volume that the material undergoes at a specific temperature. Depending upon de crosslinking density, the gel develops a loose or tight network capable of more or less swelling. This behavior is shown in Figure 1 were gels with low crosslinking density (L-HG), hence having a loose network structure, exhibit the greater swelling degree of the set of gels. This is a typical behavior as reported by Peppas and Khare.[16] Gels synthesized with the same amount of initial monomer concentration and different amounts of crosslinker (M-HG and H-HG) show similar relative swelling, however the trend on the network structure-property relationship is maintained. The collapsing temperature shows variation of about 1 °C depending upon the crosslinking density.

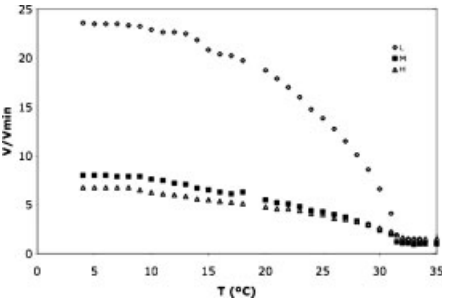

Figure 1.
Relative swelling of pNIPA hydrogels in fermentation broth as a function of temperature for several crosslinking densities: low (L), medium (M) and high (H).

The degree of swelling (V/Vmax) of pNIPA hydrogels, defined as the ratio of the volume at any temperature (V) to the maximum volume the gel reaches (Vmax), is shown in Figure 2 as a function of temperature and swelling medium for several crosslinking densities. It is interesting to notice that regardless of the kind of

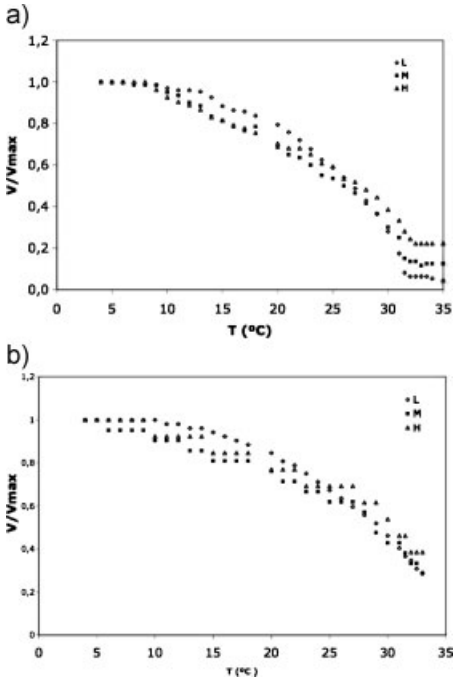

Figure 2.
Degree of swelling of pNIPA hydrogels as a function of temperature and swelling medium for several cross-linking densities (low (L), medium (M), and high (H)): in fermentation broth (a) and in water (b).

network structure, all the curves are close in the swollen state for the range of temperature studied. Similar results were found to pNIPA-HG as well when the ratio was calculated as the ratio of the hydrogel mass at any temperature to the xerogel (dry hydrogel) mass.[17] It is only at the collapsed state that the curves begin to differentiate one from another for gels swollen in broth (Figure 2a). If the gels are swollen plain water, such differentiation is absent (Figure 2b).

The average degree of swelling for L-HG, M-HG and H-HG is presented in Figure 3 as a single curve for each swelling medium. Gels show similar behaviour at low temperatures where the sweling reaches its maximum value. However, as the temperature is increased and gels go into collapsed state, less swelling occurs in broth than in water. The fact that the gels behave differently in water than in broth is

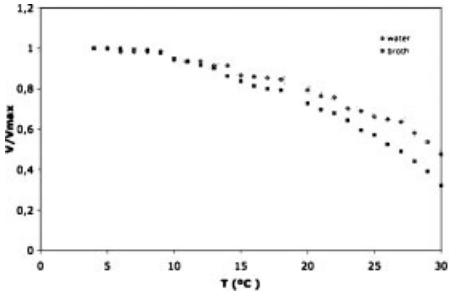

Figure 3.
Master curve for the swelling of pNIPA hydrogels in broth and in water. Each curve is the average of data presented in Figure 2.

probably due to the presence of the urea and ionic salts in the broth.[7]

Swelling Kinetics

In separation processes, it is important to know how long it takes for the gel to swell. Such information is shown in Figure 4, where the degree of swelling of pNIPA-HG is presented as function of time at different swelling temperatures for L-HG. The samples were thermostated at 33 °C prior to swelling in isothermal baths at 4 °C, 12 °C, 20 °C, and 28 °C. An estimate of the rate of swelling kinetics is obtained from the slope of these curves. The rate at which the gels swell varies as the swelling changes. At the begining of the swelling, the slope is steep and becomes smaller as time goes by. By the end, when the maximum swelling is

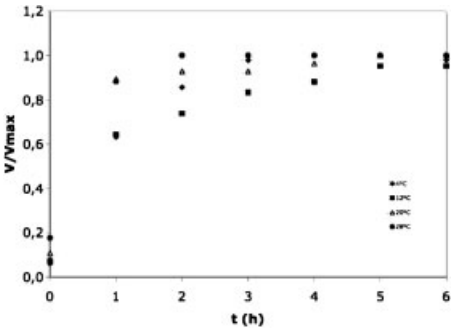

Figure 4.
Effect of the temperature on the swelling degree kinetics of pNIPA hydrogels: the samples were thermostated at 33 °C prior to swelling in isothermal baths at 4 °C, 12 °C, 20 °C, and 28 °C.

achieved, the slope is flat, and the rate of swelling is zero. This behaviour is similar regardless of the bath temperature. Differentiation of the rate of swelling for each kind of gel is made only at intermediate times when the swelling process has its own pace, depending upon the temperature gradient and is consistent to data shown in Figure 1.

The effect of the network structure on the swelling kinetics of the gels is shown in Figure 5, where the degree of swelling of pNIPA-HG is presented as function of time for several crosslinking densities: low (L), medium (M) and high (H). The samples were thermostated at 33 °C prior to swelling in an isothermal bath at 4 °C. Likewise the previous results, the same trend is observed regardless the crosslinking density. Differentiation of the rate of swelling for each kind of gel is made only at intermediate times when the swelling process has its own pace, depending upon the network structure.

Recovery and Separation

A concentrating factor (CF) was defined for the hydrogels.[12,13] In this case, the CF is defined as the ratio between the mass of the solute in the raffinate and that in the feed. Such a definition will allow one to know how much solute is being recovered with respect to the amount fed in every concentration step. The results are shown in

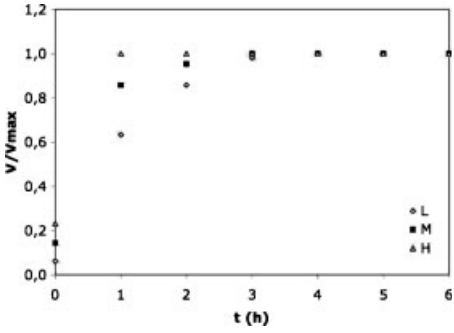

Figure 5.
Effect of the crosslinking density (low (L), medium (M), and high (H)) on the swelling degree kinetics of pNIPA hydrogels: the samples were thermostated at 33 °C prior to swelling in isothermal baths at 4 °C.

Table 1.
Concentration Factor (CF) for pNIPA hydrogels (HG) for several crosslinking densities: low (L), medium (M) and high (H).

HG type	No. of runs	Protein CF	Enzyme activity CF
		mean ± SD	mean ± SD
L-HG	11	62.2 ± 3.8	61.6 ± 11.5
M-HG	06	73.8 ± 4.8	75.8 ± 12.9
H-HG	06	75.7 ± 8.1	80.7 ± 15.6

Table 1. For the total soluble protein, the values were comparable. The protein and the enzyme activity CF for the low crosslinking density hydrogel were found to be statistically significant different (t- test) than the same factors for the others hydrogels. Considering only on the CF, the most attractive hydrogel would be the one that concentrates the largest amount of broth per unit of time. This concept deviates from the usual definition of efficiency[14] where the most efficient is the one closest to ideality. By observing the values in Table 1 and considering the amount of time involved in the separation process for each hydrogel, it can be deduced that the most promising gel would be the high crosslinking density hydrogel.

Conclusions

p(NIPA) hydrogels are good candidates as separation materials for concentrating enzyme solutions. The hydrogels show limitations in obtaining a desired concentration in a single step process. However, re-circulation of the permeate stream and multiple step preparations are options to obtain any concentration desired.

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