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Size exclusion behavior of hydroxypropylcellulose beads with temperature-dependent porosity

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Abstract

Beads prepared from a thermosensitive polymer, hydroxypropylcellulose, exhibit temperature-dependent porosity. At temperatures below 40°C the beads are swollen having large pores, while at temperatures above 45°C the beads are in a shrunken state having smaller pores. In the presence of 1 *M* NaCl the transition temperature decreased to about 30°C. In a swollen state the size of pore is large enough to accommodate lysozyme (mol. mass 14 400) and α-chymotrypsin (mol. mass 21 600) but not bovine serum albumin (mol. mass 67 000). When the beads are shrunken, all the proteins are eluted from the column packed with hydroxypropylcellulose beads in the volume close to the void volume of the column.

Keywords: Porosity, temperature dependent; Stationary phases, LC; Polymer beads; Hydroxypropylcellulose

1. Introduction

Thermosensitive polymers respond with phase transition in aqueous solution or with many fold change in hydrogel size to small changes in temperature [1]. These polymers have been used successfully in bioseparations for the development of new drug-delivery systems, biocatalysts, and biosensors [2,3]. One of the applications of thermosensitive polymers, particularly poly(*N*-isopropylacrylamide), poly-(NIPAM), was to use them for the development of chromatographic matrices with porosity regulated by temperature ([4–8] and Refs. therein). As the thermosensitive polymer used, poly(NIPAM), is not GRAS (generally regarded as safe), the main objective of the present investigation was to develop chromatographic matrices with temperature-regulated porosity using polymers with good biomedical status, e.g., cellulose derivatives.

Recent progress in biosciences and bioindustries has required new highly efficient techniques, which combine high biocompatibility, high resolution and the possibility of adjustment of the means of purification of the particular target biomolecule. The systems with regulated porosity and good biocompatibility could be of great interest for the development of new processes for the purification of biomolecules.

Cellulose ethers are known to undergo phase separation in aqueous solutions with increasing

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temperature, e.g., ethyl(hydroxyethylcellulose) [9,10] and methylcellulose [9,11] precipitate from aqueous solutions at temperatures above 40°C, while more hydrophobic (2-hydroxy-3-butoxypropyl)cellulose precipitates at temperatures just above 20°C [11]. As the cellulose ethers are the derivatives of natural polymers, the variation between products of different manufacturers and even between batches of the same trademark could be quite significant in determining different behaviours of the polymer solutions in response to temperature. For example, in another study [12] methylcellulose was reported to form a gel, rather than precipitate, when aqueous polymer solution was heated above 20°C. Hydroxypropylcellulose (HPC) in concentrations less than 40% is reported to phase separate at 44±2°C, while HPC solutions of higher concentration form a gel when heated [13]. HPC belongs to the GRAS polymers and is used in a wide variety of applications, including food, cosmetics, pharmaceuticals, coatings, adhesives, extrusions and mouldings, paper, paint removers, encapsulations, inks and many other applications requiring a film-forming agent, thickener, stabiliser, suspending agent, or protective colloid [14]. The preparation of microporous HPC gels [15] and HPMC gels [16] have been reported.

We have chosen HPC with molecular substitution 4.5 (Klucel type GF-EP, Hercules), a polymer accepted in the European Pharmacopaea, in order to produce polymer beads and evaluate them as a chromatographic matrix with porosity, regulated by temperature.

2. Materials and methods

2.1. Materials

Hydroxypropylcellulose, Klucel type GF-EP, was a gift from Hercules (Malmö, Sweden). Sepharose 2B and Sephadex G100 were purchased from Pharmacia Biotech (Uppsala, Sweden). Sephadex G100, supplied as a dry powder, was allowed to swell in pure water in a water bath for 5 h at 90°C. All the gels were degassed before being packed in the columns. Divinylsulfone was obtained from Sigma (St. Louis, MO, USA) and used without purification. Rapeseed oil, food grade, obtained from a grocer's shop, was used without purification. Triton X-100, scintillation grade, was from Merck (Darmstadt, Germany). Blue Dextran 2000 was from Amersham–Pharmacia Biotech (Uppsala, Sweden). Lysozyme and bovine serum albumin fraction V were purchased from Sigma. Chymotrypsin was purchased from ICN Biomedicals (OH, USA). Deionized water was used for preparation of sample solutions and mobile phases.

2.2. Synthesis of hydroxypropylcellulose beads

Ten grams of Klucel were mixed with 150 ml water. The resulting mixture was agitated overnight for complete dissolution of the polymer. A cross-linking agent consisting of sodium hydroxide (3.3 ml, 3.8 *M*) and divinylsulfone (4.0 ml) was added to the agitated polymer solution. Agitation was discontinued and 300 ml rapeseed oil added. Agitation was continued to obtain a dispersion of the polymer solution in the oil phase. Cross-linking was allowed to continue overnight. The resulting dispersion was mixed with water containing Triton X-100 (300 mg/ml). The formed beads were allowed to sediment down into the water phase and were washed several times with water containing Triton X-100. Beads were also subjected to several cycles of heating from room temperature to 80°C. Finally the beads were sieved at room temperature on stainless steel screens and fractions between 100 and 300 μm were collected.

2.3. Characterization of the hydroxypropylcellulose beads

The turbidities of the polymer solution (1 mg/ml) in water and in the presence of 1 *M* NaCl at various temperatures were measured at 470 nm using an Ultrospec 1000 thermostated spectrophotometer (Pharmacia Biotech) connected to a Grant waterbath.

Compression–expansion of HPC beads was studied as follows. HPC beads were packed into a 20×1-cm I.D. jacketed column (Amersham–Pharmacia Biotech) to 14 cm bed height, with 20 m*M* phosphate, pH 7.2, as a mobile phase. The column temperature was gradually increased from 20 to 80°C. After reaching maximum compression, the gel was submitted to a gradual decrease in temperature.

The variation in volume for each temperature was registered after reaching an equilibrium value. Gentle stirring was required for an even compression and expansion of the gel. The same experiments were performed at different concentrations of NaCl.

The flow resistance of a jacketed Pharmacia column 12×1-cm I.D. packed with either HPC beads, or Sepharose 2B or Sephadex G100 was evaluated at flow-rates of 0.15–2.4 ml/min (peristaltic pump Alitea C4V) at room temperature and at 50°C. The pump was calibrated in the absence of back pressure establishing the dependence of the pumping speed on the pump settings. The water was pumped into the column from the top at a given flow-rate (as judged by the pump setting) and collected in a cylinder at the outlet on the bottom of the column. In a separate experiment the pump readings were calibrated against flow-rate without the column.

The column efficiency of the Pharmacia column from 7- to 10×1-cm I.D. packed with HPC beads, Sepharose 2B and Sephadex G100 was determined by injecting acetone 1% dissolved in pure water or 1 *M* NaCl (v/v) at an eluent flow-rate of 0.3 ml/min. The sample volume injected was less than 2% of the column volume. The elution profile was registered at room temperature and at 50°C using a Uvicord S (LKB, Bromma, Sweden) and a recorder (Pharmacia Fine Chemical Integrator) and height equivalent to a theoretical plate, HETP, was calculated as follows:

$$\mathrm{HETP} = \frac{L}{5.54\left(\frac{t_R}{W_{1/2}}\right)^2}$$

where L is the column length, t_R is the retention time and $W_{1/2}$ is the width of the band at half-height.

Temperature-dependent pore size of HPC beads was studied in 1 *M* NaCl as a mobile phase using bovine serum albumin (BSA, mol. mass 67 000), α-chymotrypsin (mol. mass 21 600) and lysozyme (mol. mass 14 400). The three probe molecules were eluted separately at a flow-rate of 0.17 ml/min at room temperature, 30 and 35°C. The elution profiles were registered at 280 nm. The void volumes of each column packed at the different temperatures were determined separately using Blue Dextran, a high-molecular mass marker.

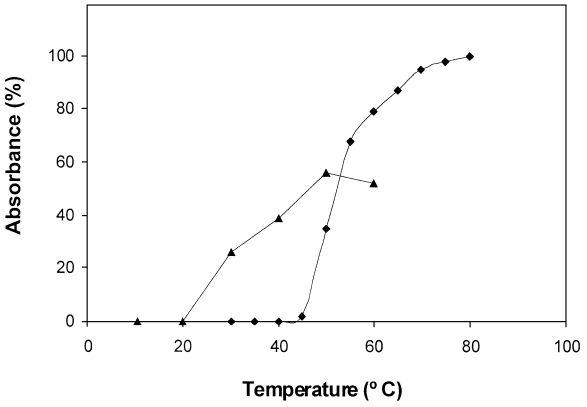

Fig. 1. Temperature dependence of the absorbance at 470 nm of aqueous HPC solution (5 mg/ml) in the absence (closed rhombs) and in the presence of 1 *M* NaCl (closed triangles). The maximal value of the absorbance of HPC solution in water was taken as 100%.

3. Results and discussion

HPC in aqueous solutions undergoes phase transition with increasing temperature.

The phase separation started around 42°C in pure water. Increasing salt concentration promoted hydrophobic interactions and in the presence of 1 *M* NaCl the phase transition occurred at lower temperatures starting approximately at 20°C (Fig. 1).

HPC beads produced by chemical cross-linking of soluble polymer responded to temperature changes

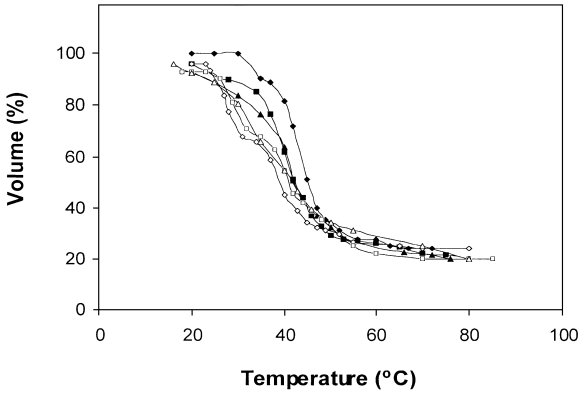

Fig. 2. Temperature dependence of volume for HPC beads. Temperature increase; first cycle (close rhombs), second cycle (closed squares) and third cycle (closed triangles). Temperature decrease; first cycle (open rhombs), second cycle (open squares) and third cycle (open triangles).

by changing their degree of swelling (Fig. 2). The transition temperature (when the response is half maximal) was about 45°C when the temperature of the swollen gel was increased or about 40°C when the temperature of the shrunken gel was decreased. The shrinking/swelling cycle is characterised by some hysteresis as the degree of swelling when temperature is increased, is always higher as compared to that when shrunken HPC beads were allowed to swell in response to decreasing temperature. The transition between swollen and shrunken state is reasonably fast and it is completed within 40 min (Fig. 3). The reproducibility of the shrinking/swelling cycle is fairly good suggesting that this process is thermodynamically controlled and results in no chemical destruction of the beads. Low salt concentration has negligible effect on the transition temperature, while in the presence of 1 *M* NaCl the transition temperature decreased to about 30°C.

The degree of swelling of HPC beads changed about 5-fold within 10–15°C temperature interval. Thus HPC beads could constitute a chromatographic matrix with a porosity which depends strongly on temperature. The behaviour of the column packed with HPC beads has been studied in detail in comparison with well-known and widely used matrices for gel filtration, Sepharose 2B and Sephadex G100. Sepharose 2B is a non-cross-linked agarose gel, which is formed spontaneously when a hot solution of agarose is cooled, while Sephadex G 100 is prepared by cross-linking dextran with epichlorohydrin (according to manufacturer's information).

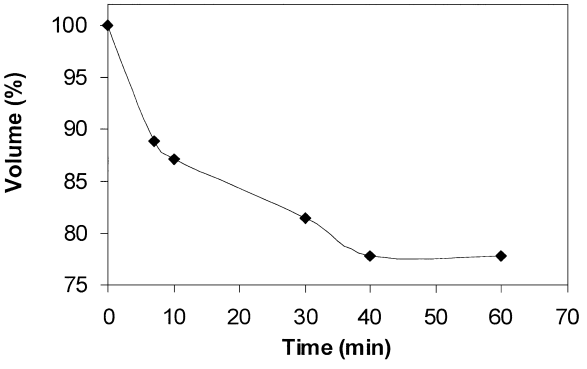

Fig. 3. Time dependence of volume change of HPC beads for temperature increase from 50 to 55°C.

At low flow-rates through the packed column, the flow should increase linearly with increasing pumping speed. As the flow-rates become higher the support packed into the column presents a resistance against the flow that leads to a decrease in flow coming out of the column. As the pumping speed becomes higher the flow that comes out from the outlet decreases, until it reaches a plateau equivalent to the maximum flow-rate. Fig. 4 shows the flow-rate profiles for the three supports at room temperature and at 50°C. Sepharose 2B is a rather soft gel, and therefore the flow-rate started to deviate from linearity due to the building up of back pressure already at relatively low pumping speeds (presented as pump

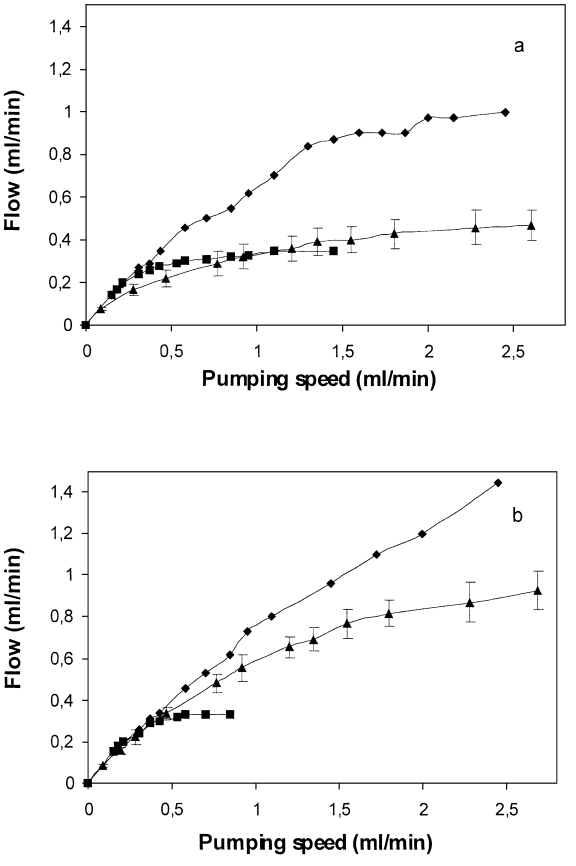

Fig. 4. Maximum flow-rates at room temperature (a) and at 50°C (b) for hydroxypropylcellulose (closed rhombs), Sepharose 2B (closed squares) and Sephadex G100 (closed triangles). Pumping speed means the settings of the pump converted into ml/min using a calibration curve obtained in the absence of back pressure.

settings converted into ml/min using a calibration curve obtained in the absence of back pressure) and levelled off at a low value of maximum flow-rate both at room and elevated temperatures. Interestingly, maximum flow-rate through Sephadex G100 column at 50°C increased considerably despite no remarkable changes were observed visually in the gel as temperature varied. As expected, maximum flow-rate though the column packed with HPC beads changed significantly with the increase in temperature. Even at low temperature, HPC beads are able to sustain much higher flow-rates than both Sepharose 2B and Sephadex G100. The difference is even more pronounced at elevated temperature. The flow-rates increase linearly with pumping speed in the whole interval studied for the column packed with shrunken HPC beads.

A general measure of chromatographic efficiency is the plate height. The smaller the plate height the more efficient is the separation process. All three gels at room temperature presented high efficiency (Table 1). The higher HETP value for Sephadex G100 at room temperature may be explained by larger bead size for this matrix. When repeating the experiments at 50°C hydroxypropylcellulose showed poor efficiency. As a rule of thumb, a HETP value for an efficient chromatographic matrix is about two to three times the size of the mean bead. For a 60–200-μm Sepharose 2B bead particle packing, this means an HETP value of 100–600 μm. The same calculations for Sephadex G-100 beads (dry bead size, 40–120 μm; swelling factor, 15–20 ml/g) results in HETP values of 200–900 μm. HPC beads with 100–300 μm size should have HETP values of 200–900 μm). Thus, in terms of chromatographic efficiency, HPC beads are comparable with Sephadex G-100 or Sepharose 2B.

The chromatographic efficiency of Sepharose 2B was not affected by changing temperature from room temperature to 50°C, while the temperature increase resulted in improved efficiency of Sephadex 100. Opposite to what was expected, the HETP values for HPC beads increased with increasing temperature despite the decrease of bead size at elevated temperature. The same effect, decrease of HETP values for HPC beads in response to increasing temperature, was observed also in the presence of 1 *M* NaCl. Probably decreased efficiency (higher HETP values) at elevated temperature was due to uneven shrinkage of HPC beads resulting in more irregular shapes and size distribution of the beads at these conditions.

The effect of temperature on the porosity of HPC beads was evaluated using probe molecules with different molecular weights. As 50°C could be hardly acceptable for working with proteins, the experiments were performed in the presence of 1 *M* NaCl, at the conditions where the shrinkage of HPC beads takes place in an acceptable temperature range of 23–35°C.

At room temperature Blue Dextran and BSA (mol. mass 67 000) were eluted from HPC beads column at the same time in the void volume, while α-chymotrypsin (mol. mass 21 600) and lysozyme (mol. mass 14 400) were retained indicating that that these molecules could diffuse inside the beads. Almost the same elution pattern was observed at 26°C. At 30°C HPC beads started shrinking and the retention time for α-chymotrypsin and lysozyme decreased, still both proteins were retained by the column. At 35°C, the HPC beads were completely shrunken (in the presence of 1 *M* NaCl) and all the proteins were eluted in the volume close to the void volume of the column. Thus, the pores of the HPC beads at room temperature could accommodate proteins with molecular mass smaller than 20 000, while at elevated temperatures, shrunken HPC beads

Table 1
Efficiency of Sepharose 2B, Sephadex G100 and HPC columns detected by injecting 1% acetone pulse

	Sepharose 2B		Sephadex G100		HPC			
	RT	50°C	RT	50°C	RT	50°C	RT NaCl 1 M	35°C NaCl 1 M
HETP (μm)	300	250	1000	500	700	1000	350	900

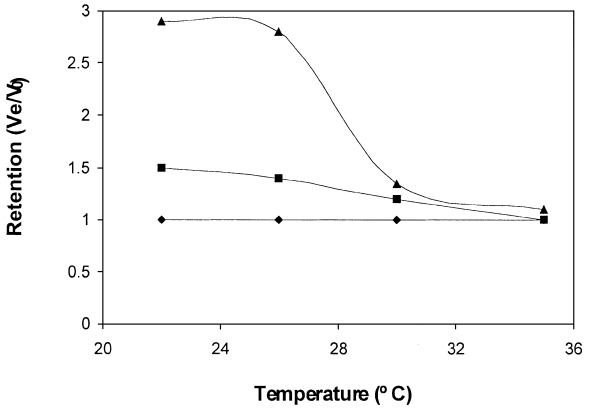

Fig. 5. Comparison of the retention at different temperatures for lysozyme (closed triangle), chymotrypsin (closed square) and BSA (closed rhomb). V_e is the elution volume of the probe molecule and V_o the void volume.

exclude even proteins as small as 14 000 molecular mass. The retention for the three probe molecules is demonstrated as a function of temperature in Fig. 5.

The developed preparation of HPC beads is far from being optimised with regard to the properties of soluble polymer or the process of bead production. One could imagine using more hydrophobic cellulose derivatives or using hydroxypropylcellulose with higher degree of substitution expecting transition to take place at temperatures more acceptable for separation of such fragile molecules as proteins. On the other hand optimisation of bead size and cross-linking density could result in beads with changes in pore size in a much broader interval as compared to the presented HPC beads.

HPC beads present a kind of temperature-controlled chromatographic matrix with properties that could be changed to a significant degree. One may simply adjust the temperature to control the maximum pore size of the beads to allow the unwanted, high-molecular-mass substances to be eluted in the void volume. One could imagine that temperature-controlled chromatographic matrices could alleviate the necessity of using several gel packing media for different pore sizes. A temperature-controlled column would also allow dynamic protein separation using controlled thermal programming along the column.

4. Conclusions

The uniformly sized hydroxypropylcellulose beads were prepared and shown to have temperature-dependent porosity. The beads could be used as a gel filtration chromatographic matrix with separation properties regulated to a high degree by changing temperature.

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