Preparative SEC Column Packed with Microporous Particles Prepared from Cellulose

Xiaopeng Xiong and **Lina Zhang***
Department of Chemistry, Wuhan University, Wuhan 430072, China

Abstract

New microporous particles with large pore size (mean pore diameter of 820 nm) are successfully prepared from a mixture of cellulose and konjac glucomannan (RC–KGM3) in 1.5M NaOH–0.65M thiourea aqueous solution by coagulating with 5 weight percentage (wt%) $CaCl_2$, and then 2 wt% HCl aqueous solution. A preparative size-exclusion chromatographic (SEC) column packed with the gel particles is used for the fractionation of a dextran in water. The exclusion limit and fractionation range of the stationary phase are molecular masses of 125×10^4 g/mol and 5.6×10^4 to 125×10^4 g/mol, respectively. The dextran [dextran 50, weight-average molecular mass (M_w) = 40.1×10^4 g/mol, polydispersity index (d) = 3.5] is fractionated by the preparative SEC column to obtain six fractions, and four of them are refractionated twice by the same preparative SEC column. The refractionated samples F-3-3 and F-4-3 are characterized by analytical SEC combined with laser light scattering and light scattering to obtain M_w of 91.8 and 61.9×10^4 g/mol, as well as d of 1.3 and 1.4, respectively. The results indicate that the fractions having narrow molecular mass distribution are satisfactorily prepared with the SEC column. The described SEC column can be successfully used to fractionate polymers in aqueous solution.

Introduction

Increasing attention has been focused on the preparative fractionation of water-soluble polymers and biopolymers by size-exclusion chromatography (SEC) in industrial process and in laboratories (1–4). The column packing materials have conventionally been made of cross-linked dextran, agarose, and polyacrylamide. Recently, various new packing materials have been developed for special applications (5–9), such as porous solid particles filled with dextran gel (5) and cellulose-based chiral stationary phases for separation of racemates (8,9). Moreover, many packings, columns, and column technology have been reviewed (2,10).

Cellulose, a renewable and environmentally friendly material, is the richest and the oldest natural polymer on earth, and this polymer can be regenerated or derivatized to yield various useful products as a results of its renewability, biodegradability, biocompatibility, and derivatizability (11). Cellulose has been used as a chromatographic packing material (12), support for immobilization and chromatographic purification of proteins (13), and ion-exchange or affinity chromatography. Commercial regenerated cellulose gels in a bead shape, used for the separation and purification of polysaccharides and proteins, suffer from the disadvantage of high cost or limited fractionation range. Therefore cellulose-based packings have been studied in details (14–16). In our laboratory, regenerated cellulose gel particles have been prepared to use as packing of preparative SEC columns (17,18), which have been successfully used to fractionate polysaccharides in water or in dimethylsulfoxide (DMSO) (17–20). However, the exclusion limit of the stationary phase is 70×10^4 g/mol, and, therefore, it is not suitable for the fractionation of macromolecules with high molecular mass. More recently, microporous membranes having large pore diameter and wide pore-size distribution have been obtained from a mixture of cellulose and konjac glucomannan (KGM) in 1.5M NaOH–0.65M thiourea aqueous solution, a new solvent of cellulose, in our laboratory (21). It has provided a simple and cheap technology that is useful for the preparation of novel cellulose gel particles. In the present work, an attempt was made to prepare regenerated cellulose gel particles with larger pore size and wider pore-size distribution. An attempt to use them in a preparative SEC column was then made. The properties of the packings and the column were investigated, and the fractions isolated with the preparative SEC column were characterized by analytical SEC combined with laser light scattering (LLS) and light scattering (LS).

Experimental

Preparation of cellulose gel particles

The cotton linter was supplied by Hubei Chemical Fiber Group, Ltd. (Xiangfan, China), and the viscosity average molecular mass of the linters in cadoxen at 25°C was determined, by viscometry, to be 10.1×10^4 (22). Cadoxen is a kind of solvent of cellulose, namely a 29-weight percentage (wt%) aqueous solution of

* Author to whom correspondence should be addressed: email lnzhang@public.wh.hb.cn.

ethylenediamine was saturated with CdO at 0°C under vigorous stirring and stored for 8 h below 5°C. KGM, a natural polysaccharide composed of β-1,4 pyranoside-bond-linked mannose and glucose, was supplied by Zhuxi Konjac Institute (Hubei Province, China). Cotton linters and KGM were, respectively, dissolved in 1.5M NaOH–0.65M thiourea aqueous mixture solution according to this method (21). The resulting cellulose solution was added to the KGM solution to produce mixtures having a weight ratio (w/w) of 100:0 to 60:40. The mixtures were vigorously stirred at room temperature for 1 h and degassed at 5°C. The solution of the resulting mixture was spun into 5 wt% $CaCl_2$ aqueous solution as a coagulation bath for 10 min with a syringe, then neutralized by aqueous 2 wt% HCl for 10 min at room temperature to obtain transparent fibers. The clear fibers were cut into small particles, as the packing material had lengths of 0.5–1.5 mm. They were then washed with water and coded as RC0 (100:0), RC–KGM2 (80:20), RC–KGM3 (70:30), and RC–KGM4 (60:40), respectively. They were stored in a 20% isopropanol–2% formaldehyde aqueous solution.

The volume (V) of the wet particles was measured with a glass dilatometer. The backbone density (ρ_g, density of regenerated cellulose), mean pore volume (V_p), and porosity (P_r) were measured and calculated according to the literature (17). Scanning electron micrographs (SEM) were observed on a Hitachi S-570 microscope (Hitachi, Tokyo, Japan). The wet fibers were frozen in liquid nitrogen, fractured immediately, and then vacuum-dried. The fractured surface (cross-section) of the fibers were coated with carbon and gold and then observed and photographed.

Preparation of fractions

Gel particles of RC–KGM3 were suspended in distilled water and then packed in a glass column (600 × 20 mm) to form a 500-mm length gel bed. Distilled water was used as the elution phase, and the flow rate was adjusted to 2.5 mL/min during the runs to stabilize the column for 1 week. The resulting preparative SEC was equipped with an automatic fractionation collector and peristaltic pump. Acetone was used to determine the theoretical plate number (N) of the preparative SEC column.

To prepare the fractions, dextran 50 with weight-average molecular mass (M_w) of 40.1×10^4 g/mol (Sigma, Oslo, Norway) was dissolved in distilled water to prepare a solution with a concentration of 10.0×10^{-2} g/mL. Five milliliters of the solution was injected into the column at 25°C and eluted with distilled water. The flow rate was adjusted to 2.0 mL/min during the run. The column eluate and the fractions were monitored with a UV detector (UV-160, Shimadzu, Kyoto, Japan) at 200 nm. The eluate from the preparative column was separated into six fractions according to equally covered area. The fractions were coded as F-1, F-2, F-3, F-4, F-5, and F-6, respectively. The fractions were separately collected from the eluate, rotary evaporated under diminished pressure at 37°C, and finally freeze-dried. The fractions (F-1, F-3, F-4, and F-6) were, respectively, dissolved in distilled water to prepare 2.0- × 10^{-2}-g/mL concentration solutions and refractionated by the same preparative SEC column.

Characterization of fractions

Viscosities of the fractions and dextran 50 in distilled water were measured at 25°C ± 0.1°C using an Ubbelodhe viscometer (Osaka University, Osaka, Japan). Huggins and Kraemer plots were used to estimate the intrinsic viscosity (h).

SEC–LLS measurements were carried out on a laser photometer (λ = 633 nm) (DAWN DSP, Wyatt Technology Co., St. Babara, CA) combined with a P100 pump (Thermo Separation Products, San Jose, CA) equipped with TSK-GEL G5000 and G3000 PWXL column (7.8 × 300 mm) at 25°C. A differential refractive index detector (RI-150) (DAWN DSP, Wyatt Technology Co.) was simultaneously connected. The eluent was a 0.1M NaCl aqueous solution with a flow rate of 1.0 mL/min. Astra software (version 4.70.07) was utilized for the data acquisition and analysis (Wyatt Technology).

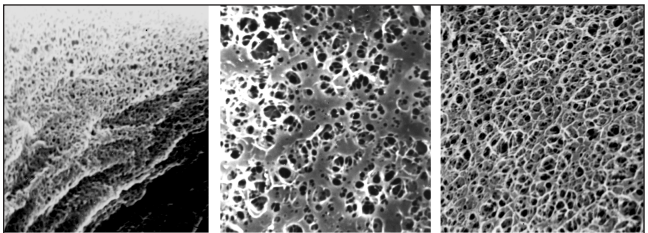
Scattering intensities of dextran 50 and the fractions in the aqueous solution were determined with the multiangle LLS instrument mentioned previously, equipped with a He–Ne laser at 25°C. Polysaccharide solutions with concentrations from 2.1×10^4 to 1.0×10^3 g/mL were prepared, and their optical clarity was achieved by filtration through a 0.2-µm pore size filter (Whatman, Oxford, U.K.) into the scattering cell (K5 mode). The refractive index increments (dn/dc) were measured with the optilab refractometer (DAWN DSP, Wyatt Technology Co.) at 633 nm and 25°C. The dn/dc value of dextran in 0.1M NaCl aqueous solutions was determined to be 0.147 mL/g.

Characterization of SEC column

The pullulan standards, which are water soluble polysaccharides, with M_w of 160×10^4 for P-160, 4.7×10^4 for P-5, and 1.0×10^4 for P-1, and fractions prepared by the preparative SEC column (M_w: F-3-3 = 91.8×10^4, F-3-3 = 61.9×10^4, F-6-3 = 30.4×10^4, and F-1-3 = 16.6×10^4 g/mol) as standard samples, whose M_w values were determined by LLS, were used here. Two milliliters of 2- × 10^{-3}-g/mL aqueous solutions of the standard samples were separately injected into the column to determine the exclusion limits of the stationary phase. The eluent was 0.1M NaCl aqueous solution with a flow rate of 0.71 mL/min. The eluate was monitored at 200 nm by UV detection.

Results and Discussion

Figure 1 shows SEM images of cross-section of the gel particles RC0, RC–KGM2, and RC–KGM3. The cross sections exhibit an obvious homogeneous pore structure. The apparent pore size increase with an increase of KGM content (Table I). When the KGM content reaches 30%, the pore size reaches the highest value. It is noted that the blended material still has good mechan-



RC0 RC–KGM2 RC–KGM3

Figure 1. SEM images of the fractured surfaces for the fiber gel RC0, RC–KGM2, and RC–KGM3.

ical properties according to previously reported results (21). The mean apparent pore diameter of RC–KGM3 was determined to be 820 nm, which is much larger than those of RC0 (140 nm) and RC–KGM2 (370 nm). It is also much larger than a value of 140 nm for RCG-1 (17), 370 nm for RCG-2 (17), and 530 nm for RCF-1 (18), which were prepared from cuoxam solution and used as the packing material for the preparative SEC column. The microporous formation mechanism holds that the voids caused by aggregation and asymmetrical shrink of KGM occurs during the coagulation process (21). The physical properties of the gel particles are summarized in Table I. The mean size of the RC–KGM3 particles at a wet state was determined to be 380 × 1240 µm (diameter x length), the pore volume was 9.87 mL/g, and the calculated porosity was 93.0%. Therefore, the RC–KGM3 was chosen as the packing materials for the preparative column because of its large pore size, wide pore-size distribution, and good mechanical property.

A general measure of chromatographic efficiency is the theoretical plate number (N). Its value of the preparative SEC column was measured to be 1120. The size-exclusion curve of the preparative SEC column (500 × 20 mm) for the standard samples in 0.1M NaCl aqueous solution, with a flow rate of 0.71 mL/min—established using the UV detector at 200 nm—(illustrated in Figure 2) is represented with the following relationship:

$$\text{Log}\, M = 3.026 - 0.190 V_e \qquad \text{Eq. 1}$$

The exclusion limit and the fractionation range of the stationary phase used in preparative SEC were obtained as M_w of 125 × 10^4 g/mol and 5.6 × 10^4 to 125 × 10^4 g/mol, respectively. Therefore, this is a good preparative SEC, having a large exclusion limit and wide fractionation range. Moreover, preparation of this

Table I. Characteristic Values of the Gel Particles

Gel	Cellulose–KGM (w/w)	Mean pore size (nm)	Backbone density (g/mL)	Pore volume (mL/g)	Porosity (%)
RC0	100:0	140	1.45	6.17	90.1
RC–KGM2	80:20	370	1.37	8.12	92.6
RC–KGM3	70:30	820	1.32	9.87	92.9
RC–KGM4	60:40	820	1.32	9.85	93.0

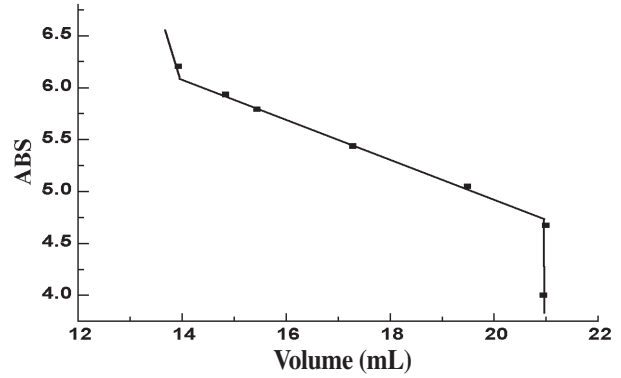


Figure 2. SEC curve of the preparative SEC column (500 × 20 mm) for pullulan standards and standard samples prepared from the preparative SEC column in 0.1M NaCl aqueous solution at 25°C, with a flow rate of 0.71 mL/min. Detector, UV at 200 nm.

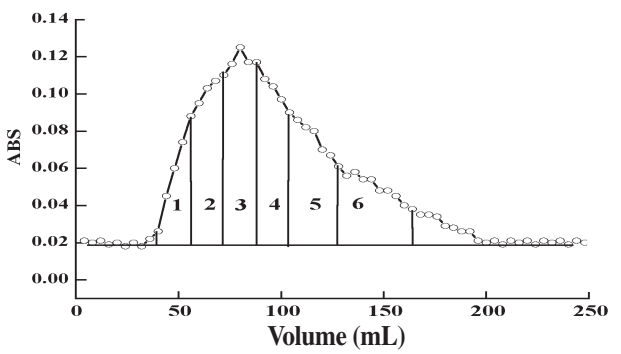


Figure 3. Elution pattern of dextran 50 measured on the preparative SEC column (500 × 20 mm) at 25°C, with distilled water as the eluent, at a flow rate of 2 mL/min. Detector, UV at 200 nm.

Table II. Experimental Results of Dextran 50 and Its Fractions

Fractionation step		(η) (mL/g)	$M_w \times 10^{-4}$ (g/mol) SEC–LLS	$M_w \times 10^{-4}$ (g/mol) LLS	Polydispersity index (d)
Dextran 50		39.5	40.1	38.2	3.5
1 Fractionation	F-1		72.5		2.0
	F-2	42.1	64.9		2.1
	F-3	39.6	57.3		2.2
	F-4	34.9	48.4		2.3
	F-5	32.3	40.3		2.4
	F-6	30.5	31.8		2.4
2 Fractionations	F-1-2		88.4		1.6
	F-3-2		91.6		1.6
	F-4-2		69.5		1.8
	F-6-2		40.5		2.2
3 Fractionations	F-3-3	57.3	91.8	85.6	1.3
	F-4-3	45.3	61.9	55.7	1.4
	F-6-3	30.1	30.4	30.1	1.8

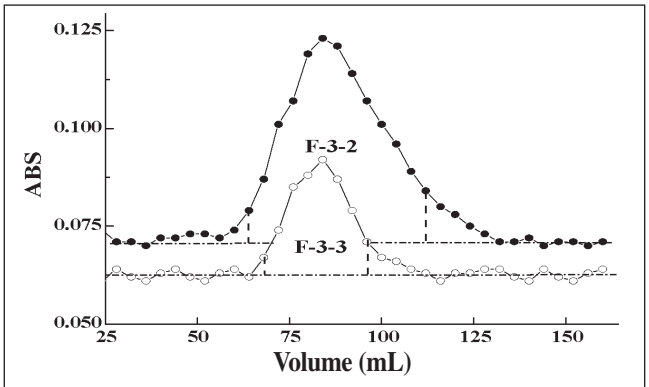


Figure 4. The second and third refractionation patterns of F-3 on the preparative SEC column (500 × 20 mm) at 25°C, with distilled water as the eluent at a flow rate of 2 mL/min. Detector, UV at 200 nm.

kind of packing is easy and simple, and the column can be used to prepare fractions of polymers.

An elution pattern of dextran 50 measured on the preparative SEC column at 25°C is shown in Figure 3. The dextran solution was injected four times, and a total of 2.01 g of dextran 50 was fractionated. The individual fractions collected from the whole injection were combined to get 0.11–0.24 g of each fraction (F-1 to F-6), and the total yield was 58%. The experimental results from viscometry, SEC–LLS, and LLS for the fractions are summarized in Table II. The intrinsic viscosities of the fractions decrease with a process of fractionation. The (η) values of F-2 and F-3 are 42.1 and 39.6 mL/g, respectively, which is slightly higher than that of dextran 50 (39.5 mL/g), although those of F-4, F-5, and F-6 are lower. The M_w of the fractions decrease from F-1 of 72.5×10^4 to F-6 of 31.8×10^4 g/mol, and the polydispersity indices (d) of the fractions are in the range from 2.0 to 2.4, which is much lower than that of dextran 50 ($d = 3.5$). The molecules are eluted out from high to low molecular mass, which is compliant with the size-exclusion principle. In view of the experiment results, the preparative SEC column is efficient for the fractionation of polysaccharides.

The elution pattern of F-3 is shown in Figure 4. The elute was collected (according to dashed line in the middle part of the refractionation curves), rotary evaporated, and freezing-dried to get F-3-3. The other three fractions were refractionated twice to get their respective fractions. F-3 was fractionated again with the preparative SEC column to obtain fraction F-3-2, and F-3-2 was refractionated to obtain F-3-3. In the procedure, only the middle part of the peak was collected, which indicates that molecules with larger and smaller molecular mass or molecular size were discarded. The SEC chromatograms of dextran 50, F-1-2, F-3-2, F-4-2, and F-6-2 are shown in Figure 5. Each chromatogram of the samples contains one peak, and the peak position of elution volumes are different, gradually shifting to a higher elution volume with the fractionation progress. The results from SEC–LLS show that M_w values of the fractions increase with a decrease of elution time. Figure 6 shows the chromatograms for fractions F-3-3, F-4-3, and F-6-3. In contrast to the second fractionation, the SEC chromatograms of these also contain single peaks and are better separated. Obviously, the d values of the fractions decrease with an increase of the refractionation times. The fractions with a d of approximately 1.3, such as F-3-3 and F-4-3, can be obtained after three subsequent refractionations. Thus, fractions or standard samples with a low polydispersity index can be prepared using this preparative SEC column. A daily throughput of 7.2 g of dextran was obtained with a flow rate of 2 mL/min and a single injection up to 0.5 g. Therefore, this work provides a simple and cheap SEC column that can be used to fractionate polymers in aqueous solution and prepare standard samples with narrow molecular mass distribution. Figure 7 shows the angular dependence of $(Kc/R_\theta)_{c=0}$ of samples of dextran 50, F-1-3, and F-3-3, in 0.1 M NaCl aqueous solution at 25°C. The M_w values determined by LLS are listed in Table II. All of the M_w values are very close to the SEC–LLS results. The results from SEC–LLS, viscosity, and LLS reveal that the preparative SEC can be used to efficiently fractionate polysaccharides in aqueous solution.

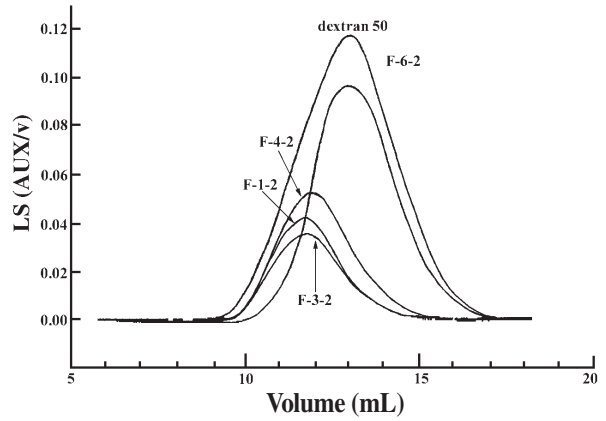


Figure 5. SEC chromatograms of dextran 50 and its twice refractionated fractions by analytical SEC–LLS.

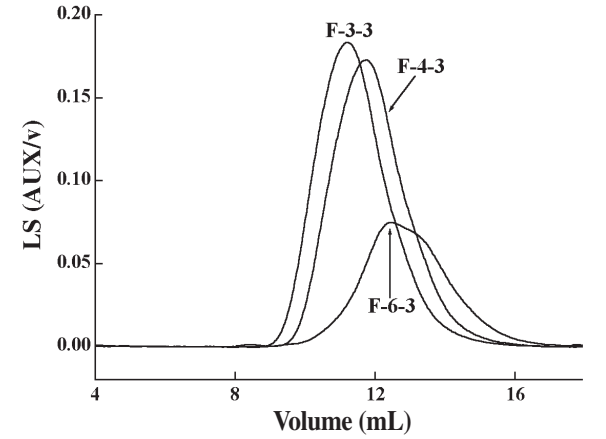


Figure 6. SEC chromatograms of the fraction obtained after three consecutive fractionations using the analytical SEC combined with LLS.

Conclusion

New cellulose gel particles RC–KGM3 with relatively large mean pore size (820 nm) were prepared from cellulose and konjac glucomannan in 1.5M NaOH–0.65M thiourea aqueous solution

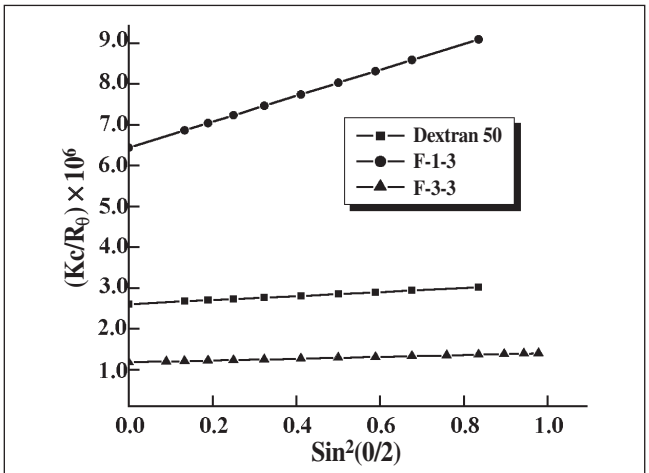


Figure 7. Angular dependence of $(Kc/R_\theta)_{c=0}$ for dextran 50, F-1-3, and F-3-3 in 0.1M NaCl aqueous solution at 25°C.

by coagulation with 5 wt% $CaCl_2$ and then 2 wt% HCl. The preparative SEC column (500 × 20 mm) packed with the particles was successfully used to fractionate dextran 50 (M_w = 40.1 × 10^4 g/mol, d = 3.5) to prepare several fractions, with M_w ranging from 16.6 × 10^4 to 91.8 × 10^4 g/mol and d of approximately 1.3 by three consecutive refractionation. The exclusion limit and fractionation range of the stationary phase were M_w of 125 × 10^4 and 5.6 × 10^4 to 125 × 10^4 g/mol, respectively. A daily throughput of 7.2 g of the dextran can be obtained with a flow rate of 2 mL/min and a single injection of up to 0.5 g. This work provided a low-cost, fast method to prepare fractions of water-soluble polysaccharides with high molecular mass and narrow molecular mass distribution on a large scale.

Acknowledgments

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References

1. B.E. Christensen, M.H. Myhr, O. Aune, S. Hagen, A. Berge, and J. Ugelstad. Macroporous, monodisperse particles and their application in aqueous size exclusion chromatography of high molecular weight polysaccharides. *Carbohydr. Polym.* **29:** 217–23 (1996).
2. P.E. Gustavsson and P.O. Larsson. Continuous superporous agarose beds for chromatography and electrophoresis. *J. Chromatogr. A* **832:** 29–39 (1999).
3. S.C. Churms. Recent progress in carbohydrate separation by high-performance liquid chromatography based on size exclusion. *J. Chromatogr. A* **720:** 151–66 (1996).
4. A.E. Rodrigues. Permeable packings and perfusion chromatography in protein separation. *J. Chromatogr. B* **699:** 47–61 (1997).
5. M. Petro, D. Berek, and I. Novák. Composite sorbents for liquid chromatography. A size exclusion study of dextran gel incorporated into porous solid particles. *React. Polym.* **32:** 2029–38 (1994).
6. Z. Ji and S. Hutt. A new bonded porous polymer PLOT U column with increased polarity. *J. Chromatogr. Sci.* **38:** 496–502 (2000).
7. S. Lei, S. Yu, and C. Zhao. Preparation of a ribonucleic acid–(polyamidoamine)–(zirconia-urea-formaldehyde resin) high-performance liquid affinity chromatographic stationary phase. *J. Chromatogr. Sci.* **39:** 280–86 (2001).
8. J. Hou, X. Han, H. Liu, Y. Wang, and J. Gao. Chiral separation of glycidyl selenide and glycidyl sulfide racemates on cellulose-based chiral stationary phases. *J. Chromatogr. Sci.* **39:** 388–92 (2001).
9. X. Chen, H. Zou, Q. Zhang, J. Ni, and Z. Zhang. Synthesis of chemically bonded cellulose trisphenylcarbamate chiral stationary phases for enantiomeric separation. *J. Chromatogr. Sci.* **40:** 315–20 (2002).
10. M.B. Baru, A.V. Danilov, and I.V. Vagenina. Size exclusion chromatography on soft and semi-rigid packing materials in the dynamic axial compression mode. *J. Biochem. Biophys. Methods* **57:** 115–42 (2003).
11. J. Schurz. 'Trends in polymer science' A bright future for cellulose. *Prog. Polym. Sci.* **24:** 481–83 (1999).
12. C. Li, C.M. Ladisch, Y. Yang, R. Hendrickson, C. Keim, N. Mosier, and M.R. Ladisch. Optimal packing characteristics of rolled, continuous stationary-phase columns. *Biotechnol. Prog.* **18:** 309–16 (2002).
13. H.F. Boeden, K. Pommerening, M. Becker, C. Rupprich, M. Holtzhauer, F. Loth, R. Müller, and D. Bertram. Bead cellulose derivatives as supports for immobilization and chromatographic purification of proteins. *J. Chromatogr.* **552:** 389–414 (1991).
14. T. Wang, Y.W. Chen, and A. Vailaya. Enantiomeric separation of some pharmaceutical intermediates and reversal of elution orders by high-performance liquid chromatography using cellulose and amylose tris(3,5-dimethylphenylcarbamate) derivatives as stationary phase. *J. Chromatogr. A* **902:** 345–55 (2000).
15. C.B. Castells and P.W. Carr. Fast enantioseparation of basic analytes by high-performance liquid chromatography using cellulose tris(3,5-dimethylphenylcarbamate)-coated zirconia stationar phases. *J. Chromatogr. A* **904:** 17–23 (2000).
16. U.J. Kim and S. Kuga. Ion-exchange chromatography by dicarboxyl cellulose gel. *J. Chromatogr. A* **919:** 29–37 (2001).
17. L. Zhang, J. Zhou, G. Yang, and J. Chen. Preparative fractionation of polysaccharides by columns packed with regenerated cellulose gels. *J. Chromatogr. A* **816:** 131–36 (1998).
18. G. Yang, L. Zhang, X. Xiong, X. Cao, and Y. Yang. Size exclusion chromatographic column packed with regenerated cellulose gels. *Chin. J. Polym. Sci.* **19:** 415–19 (2001).
19. L. Zhang, Q. Ding, D. Meng, L. Ren, G. Yang, and Y. Liu. Investigation of molecular masses and aggregation of β-D-glucan from *Poria cocos* sclerotium by size-exclusion chromatography. *J. Chromatogr. A* **839:** 49–55 (1999).
20. G. Yang, X. Xiong, and L. Zhang. Separation of superoxide dismutase by size exclusion chromatography column packed with regenerated cellulose gels. *J. Appl. Polym. Sci.* **89:** 763–68 (2003).
21. G. Yang, X. Xiong, and L. Zhang. Microporous formation of blend membranes from cellulose/konjac glucomannan in NaOH/thiourea aqueous solution. *J. Membr. Sci.* **201:** 161–73 (2002).
22. D. Ruan, L. Zhang, Z. Zhang, and X. Xia. Structure and properties regenerated cellulose/tourmaline nanocrystal composite films. *J. Polym. Sci.: Part B: Polym. Phys.* **42:** 367–73 (2004).

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