Chromatographic Performance of Monodisperse–Macroporous Particles Produced by "Modified Seeded Polymerization." I: Effect of Monomer/Seed Latex Ratio

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ABSTRACT: In this study, the monodisperse–macroporous particles produced by a relatively new polymerization protocol, the so-called, "modified seeded polymerization," were used as column-packing material in the reversed phase chromatography (RPC) of proteins. The particles were synthesized in the form of styrene-divinylbenzene copolymer approximately 7.5 μm in size. In the first stage of the synthesis, the monodisperse polystyrene particles 4.4 μm in size were obtained by dispersion polymerization and used as the "seed latex." The seed particles were swollen by a low-molecular-weight organic agent and then by a monomer mixture. The monodisperse–macroporous particles were obtained by the polymerization of monomer mixture in the seed particles. In the proposed polymerization protocol, the number of successive swelling stages was reduced with respect to the present techniques by the use of sufficiently large particles with an appropriate average molecular weight as the seed latex. A series of particles with different porosity properties was obtained by varying the monomer/seed latex ratio. The separation behavior of HPLC columns including the produced particles as packing material was investigated in the RPC mode using a protein mixture including albumin, lysozyme, cytochrome *c*, and ribonuclease A. The chromatograms were obtained with different flow rates under an acetonitrile–water gradient. The theoretical plate number increased and chromatograms with higher resolutions were obtained with the particles produced by using a lower monomer/seed latex ratio. The separation ability of the column could be protected over a wide range of flow rates (i.e., 0.5–3 mL/min) with most of the materials tested. J Appl Polym Sci 92: 607–618, 2004

Key words: reversed phase chromatography; protein separation; HPLC; poly(styrene-divinylbenzene) particles; monodisperse; macroporous particles

INTRODUCTION

Macroporous particles in the size range of 5–20 μm have been used as column-packing materials in high performance liquid chromatography (HPLC) applications for the qualitative or quantitative analysis of biochemicals. Most of these particles were obtained in the polydisperse form by applying suspension polymerization techniques. Starting in the 1990s, "monodisperse–macroporous particles" were introduced as "new generation packing materials."[1–10] More regular flow regime in the column, low back pressure, and liquid chromatograms with higher resolutions are some of the advantages of monodisperse packing materials.[3,7,8,9] Ugelstad et al. developed an "activated swelling method" for the synthesis of compact or macroporous, monodisperse particles in the range of 1–20 μm.[1–3] El-Aasser et al. prepared monodisperse—macroporous particles 10 μm in diameter via seeded emulsion polymerization.[4,5] Frechet et al. developed a "staged shape template polymerization" for the synthesis of monodisperse–porous poly(styrene-*co*-divinylbenzene) beads 7.4 μm in size.[6,7] A "dynamic swelling method" was developed by Okubo and Nakagawa for the synthesis of compact-monodisperse latex particles.[11] A relatively new method, the "Shirasu porous glass technique," was proposed for the synthesis of styrene-divinylbenzene-based monodisperse particles in the range of 2.5–60 μm.[12,13] Recently, monodisperse, crosslinked core-shell microspheres carrying a chloromethyl group were prepared by two-step precipitation polymerization.[14]

The surface modification methods, and the chromatographic interaction of functionalized structures with the biomolecule-like proteins, glycoproteins, and nucleic acids, were extensively investigated particularly for monodisperse latex particles produced by emulsion or dispersion polymerization processes.[15–30]

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However, the number of studies related to the effect of particle properties on the performance of HPLC supports is still limited.[31–36] The porosity properties and particularly the surface chemistry are important factors controlling the nonspecific adsorption of biomolecules onto the packing-material and strongly affecting the separation behavior of the reversed phase chromatography (RPC) column. The chromatographic behavior of monodisperse–macroporous particles produced by staged shape template polymerization with different surface chemistries was investigated by Frechet et al.[31–36]

Our recent studies mainly focused on the production and characterization of monodisperse–macroporous particles with different surface chemistries.[37–40] In these studies, styrene-divinylbenzene-based particles were obtained by modified seeded polymerization in the size range of 5–10 μm. The particles were also functionalized by the introduction of functional acrylic monomers (i.e., 2-hydroxyethylmethacrylate, acrylic acid, and glycidyl methacrylate) into the polymerization recipe.[37–40] The specific and nonspecific interactions of proteins with the particles having different surface chemistries were investigated in batch fashion.[37,38]

In the present study, we investigated the chromatographic performance of monodisperse–macroporous particles produced in our laboratory by a relatively new polymerization protocol, so-called modified seeded polymerization, in protein separation by RPC.

EXPERIMENTAL

Materials

Styrene (Yarpet AS, Turkey) was distilled under vacuum. Absolute ethanol (Tekel AS, Turkey) and 2-methoxyethanol (HPLC grade, Aldrich Chem. Co. Milwaukee, WI) were used as the continuous medium components in the preparation of polystyrene (PS) seed particles by dispersion polymerization. 2,2′-Azobis-isobutyronitrile (AIBN, BDH Chem. Ltd., Poole, England) crystallized from methanol and polyvinylpyrrolidone (PVP K-30, Mr:40.000, Sigma Chemical Co., St. Louis, MO) were used as the initiator and the stabilizer, respectively, in the dispersion polymerization. Dibutyl phthalate (DBP, Aldrich Chem. Co.) was selected as the diluent in the preparation of monodisperse–macroporous particles. Divinylbenzene (including 55% *para*- and *meta*-divinylbenzene isomers, Aldrich Chem. Co.) was extracted with 5% (w/w) NaOH solution. Benzoyl peroxide (BPO, Aldrich Chem. Co.) was used as the oil-soluble initiator in the repolymerization of monomer phase in the swollen seed particles. Sodium lauryl sulfate (SDS, Sigma Chem. Co.) was selected as the emulsifier in the preparation of aqueous emulsion medium for the swelling of PS seed particles. Acetonitrile and tetrahydrofuran (THF)—both HPLC grade—were supplied from Aldrich Chem. Co. Bovine serum albumin (BSA, Cat. No. A-2153, Fraction V, MW, 67.000), lysozyme (Cat. No. L-6876, MW, 14.600), ribonuclease A (Cat. No. *R*-5503, MW, 13.700), and cytochrome *c* (Cat. No. C-2037, MW, 12.327) were used as protein standards and all were purchased from Sigma Chem. Co. Distilled–deionized water was used in all polymerizations and chromatographic experiments.

Preparation of monodisperse polystyrene seed particles

The seed latex particles were synthesized by dispersion polymerization. Typically, styrene (20 mL) was dissolved in a solution composed of ethanol (72 mL) and 2-methoxyethanol (48 mL) and PVP K-30 (2.1 g) as the stabilizer. The initiator, AIBN (0.44 g), was dissolved in the resulting homogeneous mixture by ultrasonication for 1 min. The sealed, cylindrical polymerization reactor (Pyrex, total volume:410 mL) including the polymerization medium was placed in a shaking water bath equipped with a heater and a temperature-control system. The polymerization was conducted at 74°C, with 80 cpm shaking rate for 24 h. The product was polystyrene latex particles 4.4 μm in size. The latex particles were extensively washed with water by applying a centrifugation–decantation procedure. The molecular weight of seed latex was determined in a GPC system (Shimadzu, Japan) using polystyrene standards and THF as the mobile phase.

Synthesis of monodisperse–macroporous latex particles

The monodisperse–macroporous latex particles, used as chromatographic packing materials in this study, were obtained by a modified seeded polymerization. The following method was first tried in our laboratory. In a typical synthesis, DBP (5.0 mL) was emulsified in aqueous medium (100 mL) including 0.25% (w/w) SDS as the emulsifier. For the emulsification, the mixture of DBP–aqueous SDS solution was sonicated for 45 min in an ultrasonic bath (Bransonic 200). The latex dispersion (approximately 20 mL) including PS seed particles (3.33 g) was added to the DBP emulsion. The new dispersion was stirred magnetically (400 rpm) at room temperature for 24 h for the absorption of DBP by PS seed particles. In the following step, a monomer phase composed of styrene (2.5 mL), DVB (7.5 mL), and BPO (0.375 g) was emulsified in aqueous medium (120 mL) including 0.25% (w/w) SDS by sonication for approximately 5 min. Monomer emulsion was mixed with the aqueous emulsion containing DBP-swollen seed particles. The resulting emulsion was stirred at room temperature for 24 h at 400 rpm

for the absorption of monomer phase by the DBP-swollen seed particles. At the end of this period, an aqueous solution (6.0 mL) including 20% (w/w) PVA was added into the resulting dispersion. In the next stage, the emulsion was purged with nitrogen for 5 min. Repolymerization of the monomer phase in the swollen seed particles was carried out at 70°C and 120 cpm shaking rate for 24 h. The monodisperse–macroporous particles were obtained as the product after repolymerization. In some preparations, latex particles approximately 1 μm in size were obtained as a by-product. In such cases, the fraction containing monodisperse–macroporous particles was isolated from the dispersion by applying a successive centrifugation–decantation procedure using distilled–deionized water as the dispersion medium.[9,10]

The particles isolated by centrifugation (approximately 8.0–8.5 g depending upon the preparation conditions) were washed with ethanol several times. In these washings, the particles were dispersed in ethanol (150 mL) and the particle dispersion was magnetically stirred at room temperature for 24 h. Then the particles were precipitated by centrifugation at 4000 rpm for 5 min and the solvent (i.e., ethanol) was removed by decantation. The washing was continued by the addition of fresh ethanol. For the complete removal of diluent and linear polymer, the particles were extracted with THF (150 mL) at 60°C for 12 h in a sealed batch system. For this purpose, the particles were redispersed in THF (150 mL) and a batch-extraction was performed three times in a temperature-controlled water bath shaken at 120 cpm. In the each step, the supernatant (i.e., including THF, extracted linear polymer, and DBP) obtained by centrifugation at 4000 rpm was discarded and the fresh THF (150 mL) was added onto the precipitated particles. After completion of the extraction, the monodisperse–macroporous particles were redispersed in THF (100 mL).

Characterization of monodisperse latex particles

Average size and size distribution of particles were determined by a scanning electron microscope (SEM, JEOL, JEM 1200EX, Japan). An aqueous dispersion of cleaned latex particles (about 0.1 mL) was spread onto a copper disk and water was evaporated. Dried particles were coated with a thin layer of gold (about 100 Å) in vacuum. The specimens were examined and photographed in SEM. The magnification was set to 1000× in the SEM photographs taken for the determination of average size and size distribution. The photographs were printed in 14 × 10 cm and all beads in the photographs (approximately 100 beads in each photograph) were measured and counted. Then the number average diameter (D_n) and the coefficient of variation (CV %) were calculated. The surface morphology of particles was also evaluated by the SEM photographs taken at 4000× magnification.

TABLE I
The Properties of Polystyrene Seed Latex

Polymerization yield (% w/w)	66
Average particle size (D_n, μm)	4.39
Coefficient of variation (%)	2.47
Number average molecular weight (M_n)	1.14×10^4

Transmission electron microscopy (TEM) was utilized for the examination of the bulk structure of produced particles. For this purpose, dried particles (100–200 mg) were fixed in 1% w/w aqueous OsO_4 solution and dehydrated in a graded series of alcohol and then embedded in Araldit CY 212. Thin sections were cut serially (60–90 nm) by an Ultratom (LKB, UK) and mounted on 100 mesh grids and examined under a transmission electron microscope (JEOL, JEM 1200 EX, Japan).

Chromatographic study

The monodisperse–macroporous particles were slurry packed into 300 × 7.8 mm ID stainless-steel HPLC columns. For this purpose, the particle dispersion including THF as the continuous medium was first fed into the column under a pressure of 10 atm. Excess THF was removed by pressure. When filling was completed, the column was connected to the HPLC system and pure acetonitrile (120 mL) was passed at a flow rate of 2 mL/min. In the next stage, 30:70 acetonitrile/water was pumped through the column for 30 min by adjusting the flow rate at which the pressure was 125 atm. At the end of this period, the column was separated from the system and the decrease in the height of the packed bed formed at the inlet of the column was checked. The gap was filled by the addition of fresh particle slurry. Then the column was again connected to the solvent flow. During the passing, the flow direction was periodically changed by applying pressure from both ends. The passing of 70/30 water–acetonitrile solution was continued until no change was observed in the level of the column packing at both ends under 125 atm pressure.

Chromatography was carried out using a Shimadzu gradient liquid chromatograph (LC-10 ADVP) equipped with a SPD-10 AVVP UV detector. BSA, lysozyme, ribonuclease A, and cytochrome *c* were used as the protein standards. The separation of these proteins was studied under an acetonitrile/water linear gradient at room temperature. The chromatograms were obtained at different flow rates ranging between 0.5 and 3.0 mL/min at 280 nm. Trifluoroacetic acid (TFA, Aldrich Chem. Co.) was included in both acetonitrile and water phases at a concentration of 0.15% (v/v). In the liquid chromatograms of proteins, the

TABLE II
The Production Conditions of Monodisperse–Macroporous Particles

Code	Seed Latex (g)	DBP (mL)	S (mL)	DVB (mL)	BPO (g)
TG1	1.18	1.77	2.50	7.50	0.375
TG6	2.50	3.75	2.50	7.50	0.375
TG7	2.86	4.29	2.50	7.50	0.375
TG8	3.33	5.00	2.50	7.50	0.375
TG9	4.25	6.38	2.50	7.50	0.375

resolutions of successive peaks (i.e., $R(n + 1/n)$ values) were calculated according to Eq. (1), where R is the resolution between the peak of interest (peak n + 1) and the preceding peak (peak n). t_{n+1} and t_n are the retention times measured from the point of injection for peak n + 1 and peak n, respectively. W_{n+1} and W_n are the width of the base for peaks n + 1 and n, respectively. The theoretical plate number (N_t) and the reduced plate height (h_r) were calculated from the peaks of BSA in a water/acetonitrile mixture (25:75) at different flow rates between 0.5 and 3.0 mL/min based on Eqs. (2) and (3), respectively, where t_r and t_w are the retention time and the peak width at half-height, respectively. L is the column length and D_n is the number average diameter of particles.

$$R(n + 1/n) = 2(t_{n+1} - t_n)/(W_{n+1} + W_n) \quad (1)$$

$$N_t = 5.54(t_r/t_w)^2 \quad (2)$$

$$h_r = L/N_t D_n \quad (3)$$

Size-exclusion chromatography (SEC) was performed in THF using stainless-steel columns 300 × 7.8 mm ID. Polystyrene standards with different average molecular weights (Aldrich Chem. Co., MW 1,460,000, 692,000, 288,000, 92,000, 50,000, 19,200, 4000, 2100) were utilized for obtaining SEC calibration curves. During the SEC experiments, the mobile-phase flow rate was fixed at 1.0 mL/min and the column was operated at room temperature. The chromatograms were obtained by an SPD-10 AVVP UV detector at 254 nm. The back pressure of the column, packed with the particles synthesized with a certain M/SL ratio, was determined by varying the mobile-phase (i.e., THF) flow-rate in the range of 0.25–3.0 mL/min.

RESULTS AND DISCUSSION

Characterization of seed latex

The seed latex was prepared by dispersion polymerization of styrene in ethanol-methoxyethanol medium. The properties of seed latex are given in Table I. The coefficient of variation for size distribution indicated that the seed particles were nearly uniform in size.

Characterization of monodisperse–macroporous latex particles

To have monodisperse–macroporous particles with different porosity properties, the monomer/seed latex

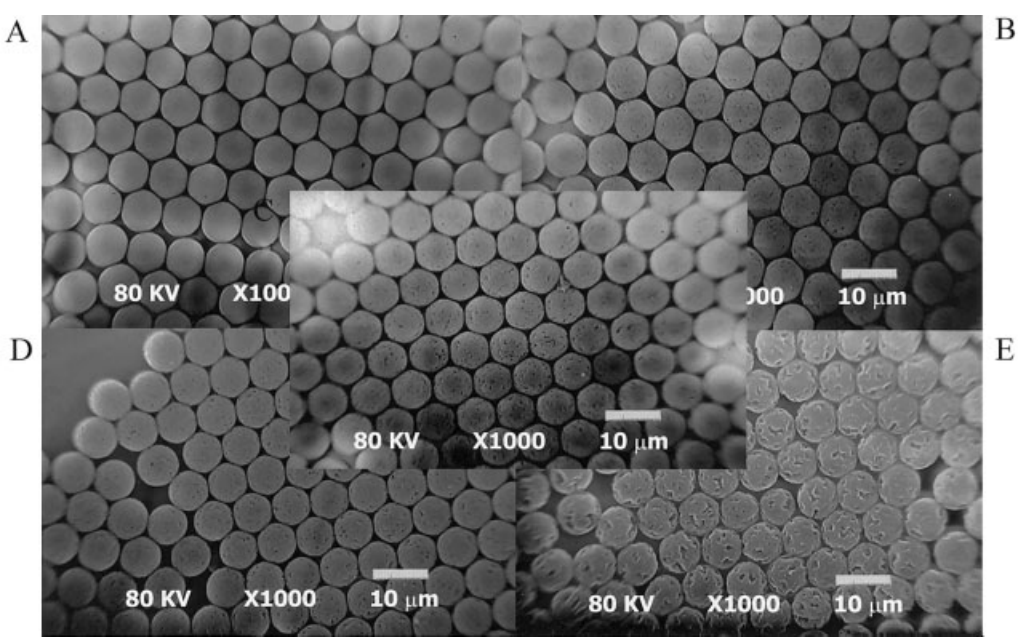

Figure 1 The SEM photographs exemplifying the monodispersity of macroporous poly(S-*co*-DVB) particles obtained with different monomer/seed latex (M/SL) ratios. M/SL ratio (mL/g): (A) 8.50, (B) 4.00, (C) 3.50, (D) 3.00, (E) 2.35 (original magnification: 1000×).

TABLE III
The Variation of Average Size and Size Distribution Properties of Monodisperse–Macroporous Particles with the Monomer/Seed Latex (M/SL) Ratio

Code	M/SL (mL/g)	D_n (μm)	CV (%)
TG1	8.50	7.15	3.58
TG6	4.00	7.10	3.06
TG7	3.50	6.80	3.82
TG8	3.00	6.71	2.24
TG9	2.35	7.00	2.57

(M/SL) ratio was varied between 2.35 and 8.50 mL/g. This change was achieved by decreasing the amount of seed latex in the presence of constant monomer volume (i.e., 10 mL). Our previous studies showed that appropriate pore structures were obtained with diluent/seed latex ratios ranging between 1 and 2 mL/g.[9,10,37–40] For this reason, the DBP/seed latex ratio was fixed at 1.5 mL/g in this study. The initiator and crosslinker concentrations in the monomer phase were 37.5 mg/mL and 75% (v/v), respectively. The detailed production conditions of monodisperse–macroporous particles are given in Table II. SEM photographs, showing the size distribution characteristics of the particles synthesized with different M/SL ratios, are given in Figure 1. The size distribution properties, calculated based on these photographs, are listed in Table III. As seen here, all CV values were lower than 5%. This result indicated that the particles produced with different M/SL ratios were nearly uniform in size. However, the particles produced with lower monomer/seed latex ratios exhibited better monodispersity (i.e., lower CV values). As seen in Table III, the M/SL ratio had no effect on the average size of the particles. In our experiments, the monomer/seed latex ratio was decreased by increasing the amount of seed latex in the presence of constant monomer volume. However, these polymerizations were performed with a constant diluent/seed latex ratio of 1.5 mL/g. For this reason, higher diluent volume (i.e., DBP) was utilized in the case of lower monomer/seed latex ratio (Table II). In the case of lower monomer/seed latex ratio, the distribution of constant monomer volume into higher number of seed particles involves a decrease in the average size. However, the particle size should be related to the final swelling ratio of the seed particles achieved before the repolymerization. In other words, both the diluent and the monomer swelling stages are effective on the final particle size. In our case, while increasing the amount of seed latex, the total volume of the organic phase (i.e., diluent + monomer) was also increased (i.e., due to the increasing amount of diluent) (Table II). The increase in the volume of total organic phase (i.e., diluent + monomer) probably prevented an appreciable decrease in the particle size with decreasing monomer/seed latex ratio.

SEM photographs showing the detailed surface morphology of monodisperse particles are given in Figure 2. As seen here, the average pore size exhibited a clear increase with decreasing M/SL ratio. In other words, while sponge-like porosity was the dominant structure on the surface obtained with high M/SL ratios, the decrease in the M/SL ratio resulted in the formation of particles with crater-like porosity. Pores

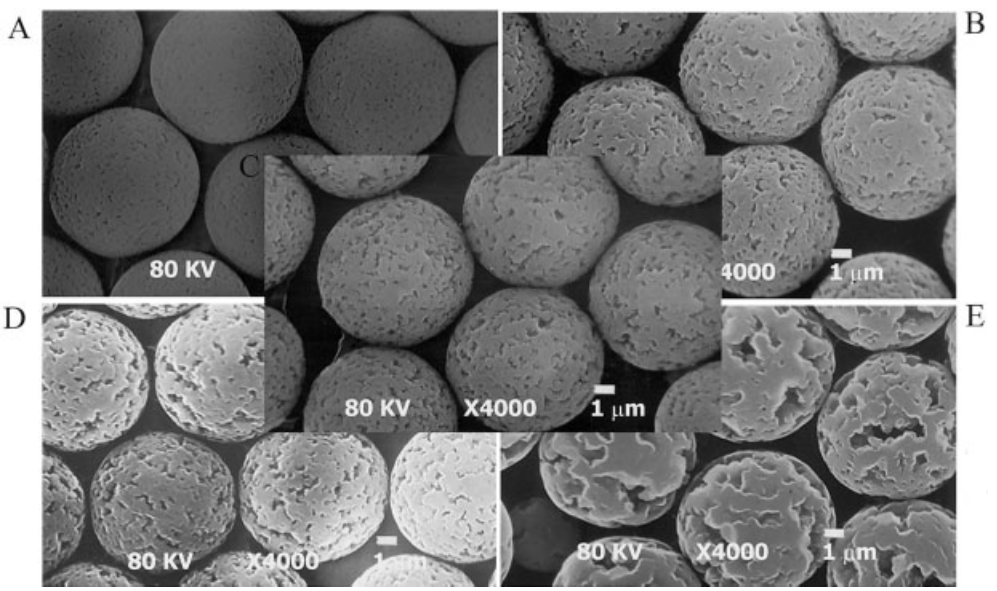

Figure 2 The SEM photographs showing the effect of M/SL ratio on the surface morphology of poly(S-*co*-DVB) particles produced with different M/SL ratios. M/SL ratio (mL/g): (A) 8.50, (B) 4.00, (C) 3.50, (D) 3.00, (E) 2.35 (original magnification: 4000×).

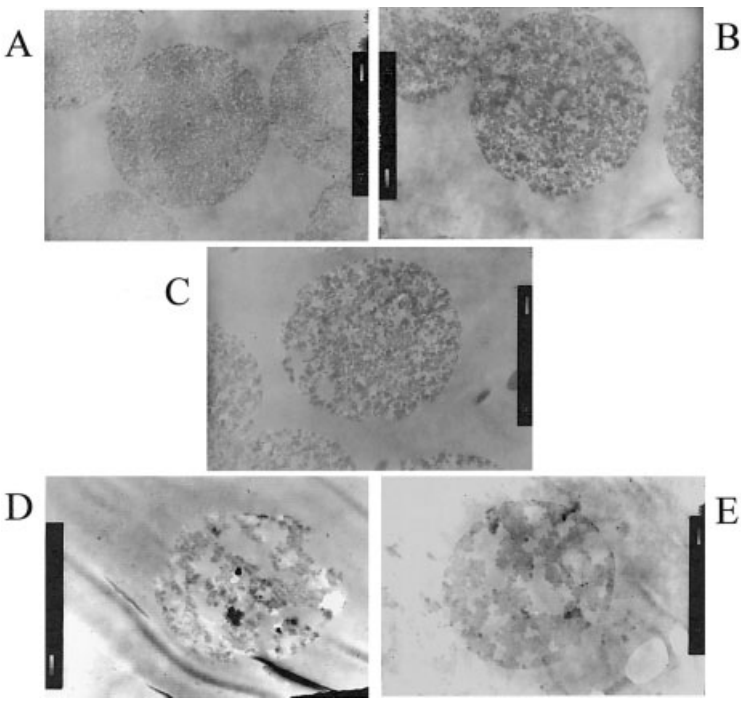

Figure 3 The TEM photographs showing the effect of M/SL ratio on the internal structure of poly(S-*co*-DVB) particles produced with different M/SL ratios. M/SL ratio (mL/g): (A) 8.50, (B) 4.00, (C) 3.50, (D) 3.00, (E) 2.35 (original magnification: 5000×).

larger than 1 μm were clearly seen on the surface of the particles obtained with the lowest M/SL ratio (Fig. 2E). To get an idea of the pore structure in the particle interior, TEM photographs of the thin crossections of the particles obtained with different M/SL ratios were taken. These photographs are presented in Figure 3. Similar to the tendency observed on the particle surface, the pore size exhibited a clear increase with decreasing M/SL ratio. As observed for the surface, the sponge-like internal structure observed with high M/SL ratio turned to the crater-like porosity with decreasing M/SL ratio. An explanation for the effect of M/SL ratio on the pore structure of monodisperse particles obtained by modified seeded polymerization was given elsewhere.[9,39,40]

Chromatographic evaluation

Monodisperse–macroporous particles, produced with different M/SL ratios ranging between 2.35 and 8.50 mL/g, were tested as packing material in reversed phase chromatography. A protein mixture, including four proteins with different average molecular weights and hydrophobicities, was used as the test sample. The chromatographic separation of the selected proteins was performed under acetonitrile–water gradient at room temperature. To determine appropriate gradient conditions for each column, a set of preliminary chromatographic separations was performed under constant gradient conditions with a flow rate of 1.0 mL/min. The columns tried and the conditions are given in Table IV. As seen here, the concentration of mobile phase A was increased from 30 to 60% (v/v) within 30 min. In other words, the slope of the gradient was fixed at 1.0 for all columns tested. The representative liquid chromatograms of

TABLE IV
The Gradient Conditions and the Resolution Values in the Separation of Proteins by Reversed Phase Chromatography under a Linear Gradient of Acetonitrile in Water with a Slope of 1.0%/min[a]

Column code	M/SL (mL/g)	Initial A (%)	Final A (%)	Time (min)
TG1	8.50	30	60	30
TG7	3.50	30	60	30
TG8	3.00	30	60	30
TG9	2.35	30	60	30

		Resolutions		
Column code	M/SL (mL/g)	R (2/1)	R (3/2)	R (4/3)
TG1	8.50	3.26	0.95	0.40
TG7	3.50	2.66	1.38	1.21
TG8	3.00	6.11	3.02	2.55
TG9	2.35	1.74	0.81	1.00

[a] Conditions: column, 30 × 7.8 mm ID; mobile phase, (A) 5% water in acetonitrile with 0.15% TFA, (B) water with 0.15% TFA; flow rate, 1mL/min; UV detection at 280 nm.

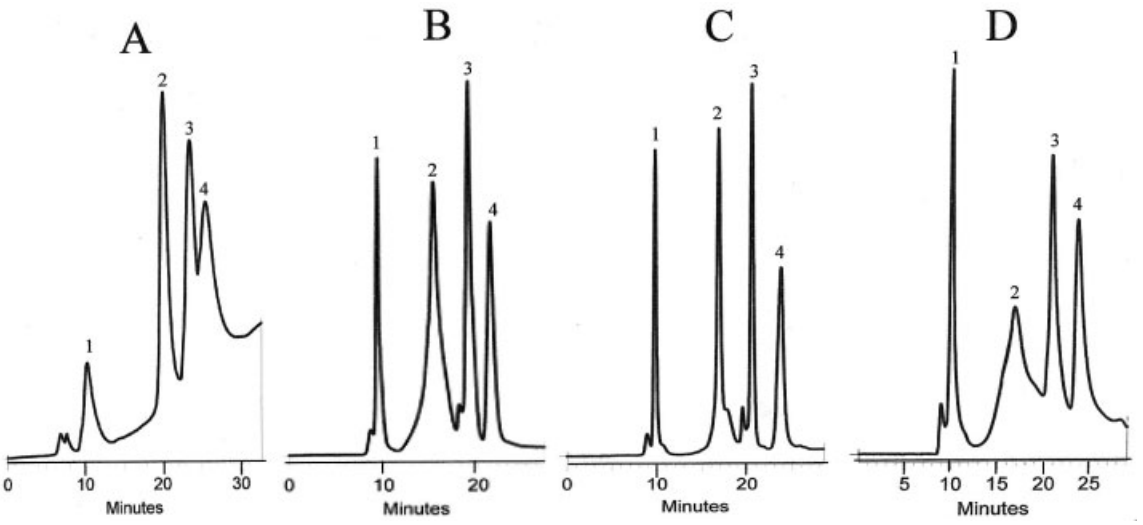

Figure 4 Separation of protein mixture on a column packed with the particles produced by different M/SL ratios under a linear gradient of acetonitrile in water with a slope of 1.0%/min. Conditions: column, 30 × 7.8 mm ID; flow rate, 1 mL/min; UV detection at 280 nm; particle properties, M/SL ratio (mL/g): (A) 8.50, (B) 3.50, (C) 3.00, (D) 2.35; elution order: (1) ribonuclease A, (2) cytochrome *c*, (3) lysozyme, (4) albumin.

the protein mixture, and the resolution values calculated based on these chromatograms, are presented in Figure 4 and Table IV, respectively. As seen in Figure 4A and Table IV, the selected protein mixture could not be separated well in the column containing the particles prepared with the highest monomer/seed latex ratio (i.e., TG1). A satisfactory separation was first observed with the column material synthesized by the monomer/seed latex ratio of 3.5 mL/g (Fig. 4B). The column prepared with the monomer/seed

TABLE V
The Gradient Conditions Used for the Separation of Proteins by Reversed Phase Chromatography under a Linear Gradient of Acetonitrile in Water with a Slope of 0.7 or 0.75%/min[a]

Column code	M/SL (mL/g)	Initial A (%)	Final A (%)	Gradient time (min)
TG1	8.50	30	60/(75)[b]	40/(60)[b]
TG7	3.50	30	55	35
TG8	3.00	30	60/(75)[b]	40/(60)[b]
TG9	2.35	30	60	40
		Resolutions		
Flow rate (mL/min)	0.5	1	2	3
M/SL:8.50 mL/g				
R(2/1)	2.68	3.35	3.69	4.84
R(3/2)	NS	1.00	1.15	1.56
R(4/3)	NS	0.72	NS[c]	NS[c]
M/SL:3.50 mL/g				
R(2/1)	2.83	3.41	4.43	2.18
R(3/2)	1.38	2.08	3.26	3.34
R(4/3)	0.73	1.87	3.18	4.84
M/SL:3.00 mL/g				
R(2/1)	5.69	9.60	10.7	14.7
R(3/2)	1.25	2.36	3.69	4.10
R(4/3)	1.20	2.13	3.20	5.91
M/SL:2.35 mL/g				
R(2/1)	3.21	3.56	3.56	1.00
R(3/2)	1.08	1.91	2.79	3.76
R(4/3)	1.23	2.08	2.96	3.26

[a] Conditions: column, 30 × 7.8 mm ID; mobile phase, (A) 5% water in acetonitrile with 0.15% TFA, (B) water with 0.15% TFA; flow rate, variable from 0.5 to 3.0 mL/min; UV detection at 280 nm.
[b] The values in parentheses are used only for the mobile phase flow rate of 0.5 mL/min.
[c] NS, no appreciable separation was observed for two successive peaks.

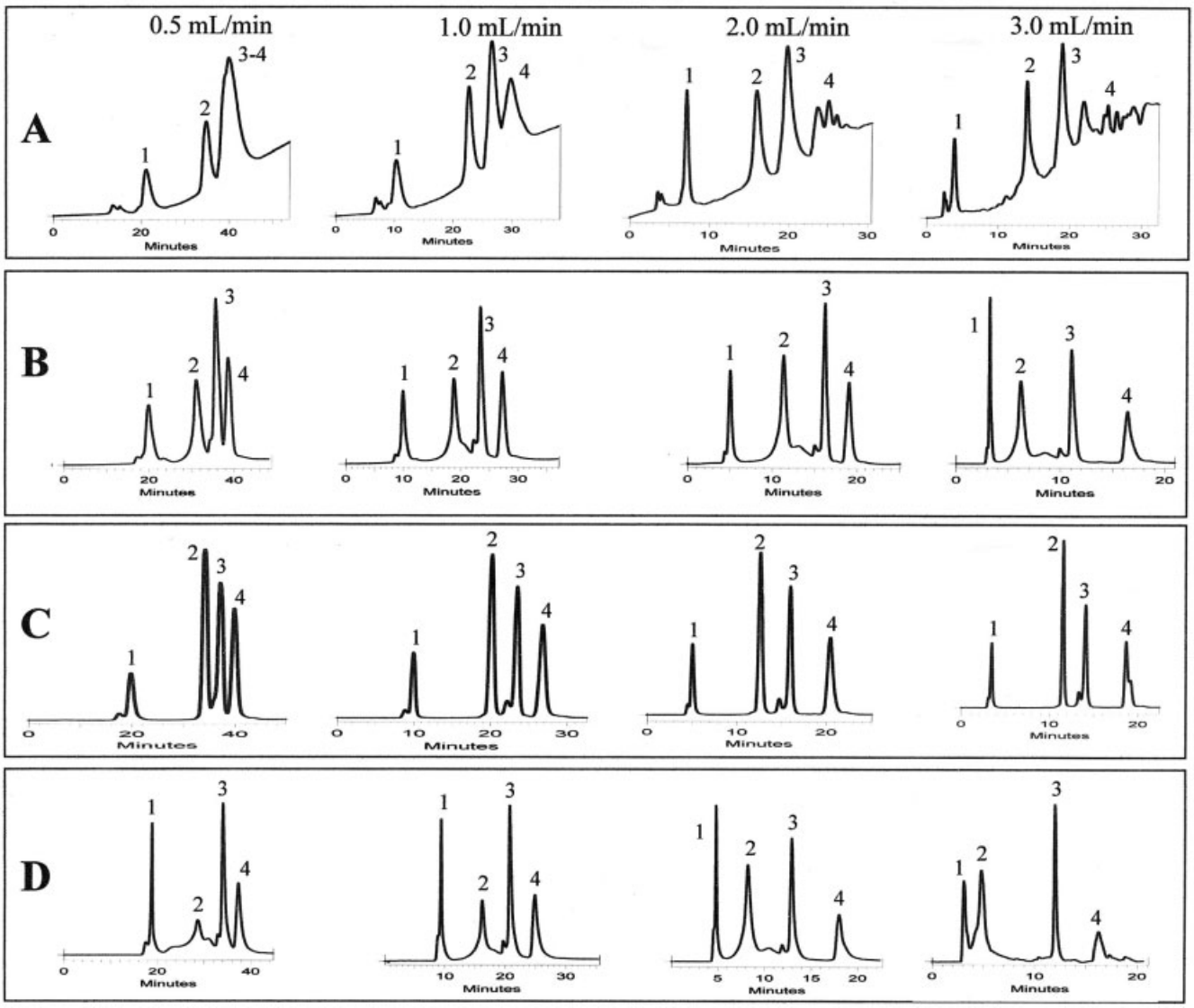

Figure 5 Separation of protein mixture with different mobile-phase flow rates on a column packed with the particles produced by different M/SL ratios. Conditions: Linear gradient of acetonitrile in water having a slope of 0.7 or 0.75%/min; column, 30 × 7.8 mm ID; flow rate, variable from 0.5 to 3.0 mL/min; UV detection at 280 nm; particle properties, M/SL ratio (mL/g): (A) 8.50, (B) 3.50, (C) 3.00, (D) 2.35. Elution order: (1) ribonuclease A, (2) cytochrome *c*, (3) lysozyme, (4) albumin.

latex ratio of 3.0 mL/g provided the best chromatographic separation (i.e., the highest resolution values) (Fig. 4C). The separation ability again decreased with column material obtained with the lowest monomer/seed latex ratio (i.e., TG9, 2.35 mL/g) (Fig. 4D).

We thought that the slope of 1%/min was too sharp for these columns and that a better separation (i.e., the resolutions comparable to those of TG8) could be obtained, particularly with TG7 and TG9, by the application of a gradient with a lower slope relative to the first one. So, the gradient starting from the same acetonitrile concentration (i.e., 30% (v/v)) and having a slope of 0.7 or 0.75%/min was tried for all columns. The conditions of this gradient are described in Table V. The liquid chromatograms recorded by varying the mobile-phase flow rate between 0.5 and 3.0 mL/min under these conditions are given in Figure 5. The resolution values calculated based on these chromatograms are also presented in Table V. Although the slope of the gradient was decreased, a succesful separation could not be achieved with all flow rates in the column prepared with the highest monomer/seed latex ratio (Fig. 5A and Table V). In this column, a significant baseline shift was also observed at all flow rates tried. The lowest resolutions, particularly for the hydrophobic proteins (i.e., lysozyme and albumin), were calculated for this column. Liquid chromatograms with satisfactory resolution values could be obtained with the columns prepared with the monomer/seed latex ratios lower than 3.50 mL/g. The rep-

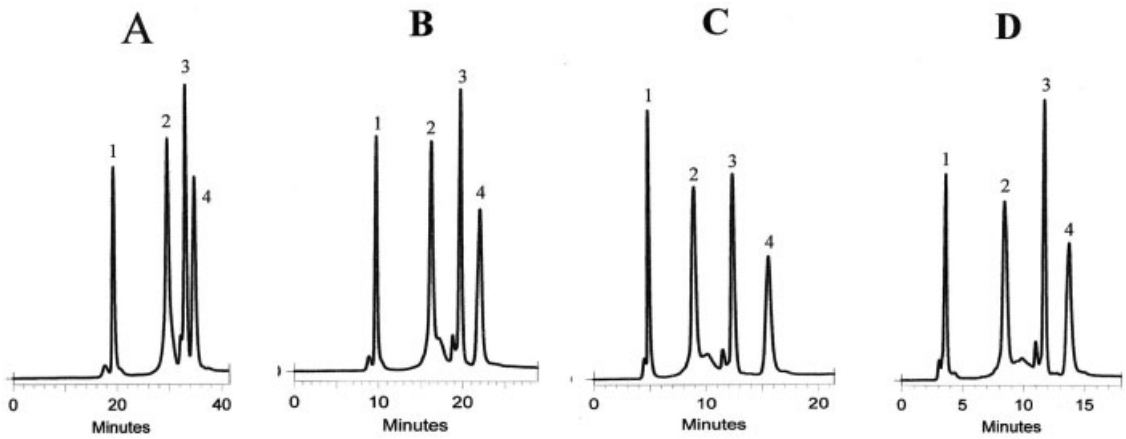

Figure 6 Separation of protein mixture on a column packed with the particles produced by the M/SL ratio of 3.0 mL/g under a linear gradient of acetonitrile in water with a slope of 1.0%/min. Column: 30 × 7.8 mm ID; UV detection at 280 nm. Flow rate (mL/min): (A) 0.5, (B) 1.0, (C) 2.0, (D) 3.0. Elution order: (1) ribonuclease A, (2) cytochrome *c*, (3) lysozyme, (4) albumin.

resentative liquid chromatograms obtained with the column-packing material prepared with the monomer/seed latex ratio of 3.5 mL/g are given in Figure 5B. As seen here, the complete separation of cytochrome *c*, lysozyme, and albumin could not be achieved with the lowest flow-rate (i.e., 0.5 mL/min.). For this reason, relatively low resolutions were found (Table V). However, the separation behavior was significantly improved and higher resolutions were obtained with the increasing flow rate (Fig. 5B and Table V). As seen in Figure 5C and Table V, the best separation behavior was observed with the column synthesized by the monomer/seed latex ratio of 3.0 mL/g. The chromatographic performance again decreased with the column prepared with the monomer/seed latex ratio of 2.35 mL/g.

The column providing the chromatograms with the highest resolution (i.e., TG8) was also tested by applying a gradient with higher slope (i.e., 1%/min) at different flow rates. The liquid chromatograms obtained under these conditions and their resolutions are given in Figure 6 and Table VI, respectively. By comparison of the chromatograms given in Figure 5C and Figure 6 at a constant flow rate, one can conclude that the retention time of ribonuclease A is not affected by the slope of the gradient since this protein is extremely hydrophilic relative to the others. However, the other proteins (i.e., relatively hydrophobic ones) were significantly retarded in the case of a gradient with lower slope (Fig. 5C). For this reason, the resolution of the first two peaks is reasonably higher at all flow rates for the gradient with a slope of 0.75%/min (Tables V and VI). At constant flow rate, the resolutions of the peaks belonging to more hydrophobic proteins obtained with a slope of 0.75%/min were not different from those obtained with 1%/min (Tables V and VI). As expected, the separation was completed in a shorter period with the gradient with higher slope at constant flow rate. (i.e., relative to the gradient with a slope of 0.75%/min).

In all cases, higher resolutions were usually obtained with higher flow rates. In the case of separation

TABLE VI
The Gradient Conditions Used for Separation of Proteins by Reversed Phase Chromatography under a Linear Gradient of Acetonitrile in Water with a Slope of 1.0%/min on the Column Packed with the Particles Produced with the M/SL Ratio of 3.0 mL/g

Column code	M/SL (mL/g)	Initial A (%)	Final A (%)	Gradient time (min)
TG8	3.00	30	60	30
		Resolutions		
Flow rate (mL/min)	0.5	1	2	3
R(2/1)	3.92	6.11	5.81	5.53
R(3/2)	1.23	3.02	4.04	3.50
R(4/3)	1.13	2.55	3.05	3.17

[a] Conditions: column, 30 × 7.8 mm ID; mobile phase, (A) 5% water in acetonitrile with 0.15% TFA, (B) water with 0.15% TFA; flow rate, variable from 0.5 to 3.0 mL/min; UV detection at 280 nm.

medium with larger pores, higher flow rate involves intraparticular, pressure-driven convective transport of the solutes in addition to molecular diffusion.[7,8,34] Then the mass transfer rate of the solute within the column increases. As seen in the SEM photographs showing the surface morphology of the particles and TEM photographs of the particle interiors, there were sufficiently larger pores, particularly in the beads prepared with the M/SL ratios lower than 3.5 mL/g. With these columns, the better separations observed for the flow rates higher than 1 mL/min should be attributed to the increasing effect of pressure-driven intraparticular convective transport (Fig. 5B, 5C, and 5D).

The effects of flow rate on the theoretical plate number and the reduced plate height are given in Figure 7. For all columns, the theoretical plate number exhibited maxima at low flow rates and then decreased with the increasing flow rate (Fig. 7A). Depending upon this tendency, the reduced plate height decreased with increasing flow rate (Fig. 7B). The maximum theoretical plate number was observed at approximately 1.0 mL/min for all columns tried. The packing material produced with the monomer/seed latex ratio of 3.0 mL/g provided the highest theoretical plate number in the flow rate range of 0.5–2.0 mL/min.

The behaviors observed for the variation of reduced plate height with the flow rate are similar to the chromatographic behavior of the particles produced with different polymerization techniques.[7,34] The efficiency of a column packed with a conventional separation medium normally decreases linearly with increasing flow rate.[7] In our case, an increase in the flow rate also involved a decrease in the column efficiency for the particles produced with the monomer/seed latex ratios of 3.0 and 3.5 mL/g (i.e., increasing reduced plate height in Fig. 7B). However, the reduced plate height of the particles obtained with the M/SL ratio of 2.35 mL/g did not change significantly with the flow rate. The mass transfer by intraparticular convection probably becomes more effective with increasing flow rate in the presence of larger pores. This case probably prevented the decrease in the column efficiency for the particles produced with M/SL ratio of 2.35 mL/g. Note that a similar effect was also observed for particles obtained with staged shape template polymerization.[7,34] Wang et al. reported that the reduced plate height of the columns packed with poly(styrene-*co*-divinylbenzene) particles produced by staged shape template polymerization was approximately between 25 and 40.[7] We also found the values varying in this range for the particles obtained by our polymerization protocol.

For the columns synthesized with different M/SL ratios, the SEC calibration curves obtained with the PS standards are given in Figure 8. Two linear regions with different slopes were observed in the calibration

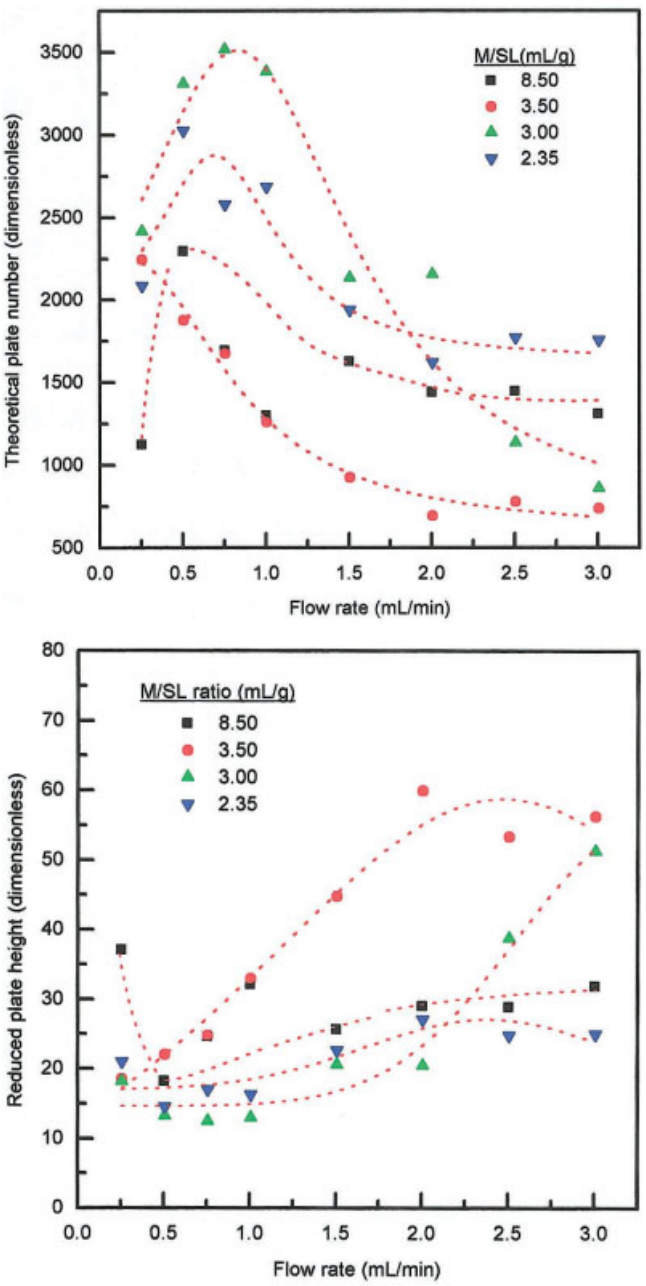

Figure 7 Effect of flow rate on the efficiency of a column packed with the particles obtained by different M/SL ratios. Conditions: column, 30 × 7.8 mm ID; Mobile phase, acetonitrile–water (60:40) with 0.15% TFA; UV detection at 280 nm; Analyte: albumin (A), number of theoretical plates, (B) reduced plate height.

curves of the particles synthesized with the M/SL ratios equal to or lower than 3.5 mL/g. In other words, the slope of the calibration curve obtained for the MWs higher than 10^5 was lower than that obtained in the MW region of 10^3–10^5. Hence, the MWs higher than 10^5 can be determined more precisely with the packing materials produced with the M/SL ratios equal to or lower than 3.5 mL/g. The evaluation of SEM and TEM photographs in Figures 2 and 3 also

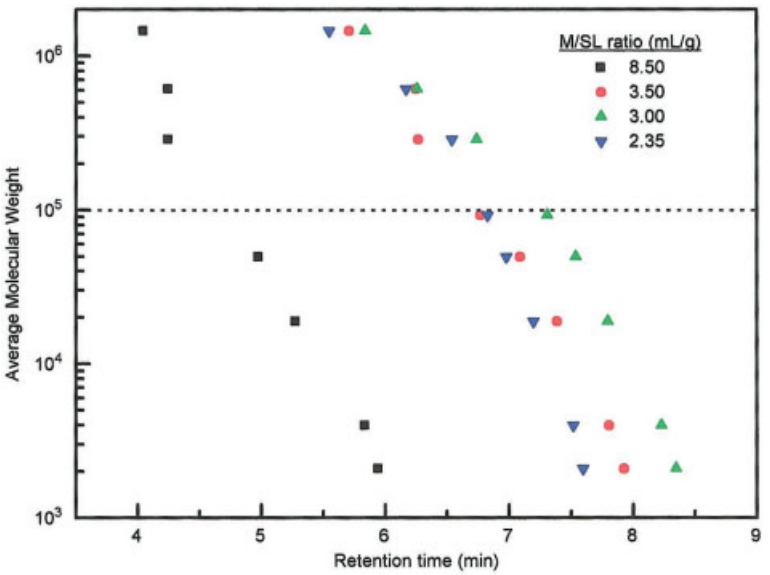

Figure 8 Size-exclusion chromatography calibration curves for polystyrene standards in THF using the columns packed with the particles obtained with different M/SL ratios. Conditions: column: 30 × 7.8 mm ID; flow rate, 1.0 mL/min; UV detection at 254 nm.

showed the presence of relatively larger pores in these particles. For this reason, a SEC calibration curve with a lower slope in the high MW region (i.e., better separation of materials with larger MWs) is an expected case for these particles. However, the packing-material synthesized M/SL ratios equal to or lower than 3.5 mL/g can separate the polymers with molecular volumes equivalent to PS standards in the range of 2.1 $\times$ 10^3 to 1.46 $\times$ 10^6. The exclusion value of the polystyrene standard with the MW of 1.46 $\times$ 10^6 was calculated as 269 nm based on the equation derived by Halasz and Martin.[41] As seen in Figures 2 and 3, the particles obtained with the M/SL ratios lower than 3.5 mL/g had sufficiently large macropores with respect to this value, which, in turn, should be suitable for the determination of MW up to—at least—1.46 $\times$ 10^6.

For the column material synthesized with the M/SL ratio of 8.50 mL/g, no significant change was observed in the retention time with the MW of the PS standard when MW was higher than 2.88 $\times$ 10^5. The exclusion value of this PS standard was 103 nm.[41] The pore structure shown in Figures 2A and 3A was also consistent with the behavior observed in the SEC curve of this material. As seen in these photographs, the pores both on the surface and in the interior were not much larger than this value. For this reason, it should be difficult to determine MWs higher than this value by using the packing material produced with the M/SL ratio of 8.50 mL/g. However, this material can be utilized for the determination of MW s lower than 10^5. On the other hand, relatively lower retention times observed in the presence of the column material synthesized with the M/SL ratio of 8.5 mL/g may be explained by the lower porosity of this material (Fig. 3A). As seen in Figure 3B–3E, the column materials obtained with M/SL ratios lower than 3.5 mL/g had significantly higher porosities and provided higher retention times.

The effect of mobile-phase (i.e., THF) flow rate on the back pressure is given in Figure 9. For all packing materials, the back pressure varied linearly with the

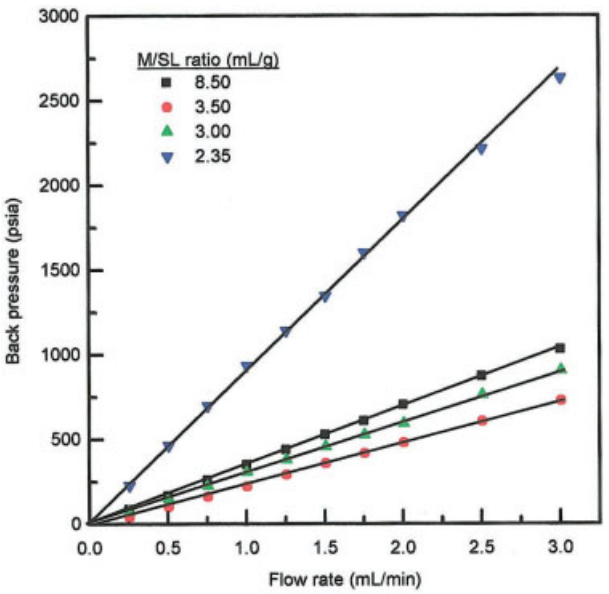

Figure 9 Effect of flow rate on back pressure of columns packed with the particles obtained with different M/SL ratios. Conditions: column, 30 × 7.8 mm ID; mobile phase, THF.

flow rate. At constant flow rate, the packing materials obtained by the M/SL ratios of 3.0 and 3.5 mL/g exhibited relatively lower back pressure. This behavior is probably explained by the higher porosity of these materials. Depending upon this tendency, the back pressure of packing material with smaller average pore size and lower porosity (i.e., the particles obtained by the M/SL ratio of 8.50 mL/g) was higher with respect to the particles obtained by the M/SL ratios of 3.0 and 3.5 mL/g. Although highly porous character of the particles produced with the lowest M/SL ratio (i.e., 2.35 mL/g) was also shown by both SEM and TEM (i.e., Figs. 2E and 3E), extremely higher back pressure was surprisingly obtained with this column. To explain this behavior, the particle samples withdrawn from the columns were examined using an optical microscope after completion of the chromatographic experiments. This examination showed that some part of the particles produced with the M/SL ratio of 2.35 mL/g were broken in the column. The low mechanical stability of the highly porous material is probably the reason for the broken particles. Hence, the breaking of particles by the pressure probably caused a significant reduction in the porosity of the column that probably led to the higher back pressures at constant flow rate. For this reason, the particles produced by the M/SL ratio of 2.35 mL/g should not be considered suitable packing materials.

CONCLUSION

In this study, the monodisperse–macroporous particles sui for chromatographic use were obtained by a relatively new and simplified polymerization protocol, modified seeded polymerization. The effects of M/SL ratio on the size and the porosity properties of final particles were investigated. In the next stage, the particles obtained with different M/SL ratios were used as packing material in the HPLC system. The RPC and SEC studies showed that the M/SL ratios of 3.0–3.5 mL/g were the most suitable values for producing HPLC packing materials by using modified seeded polymerization.

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