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Uniform Particles for the Reversed-Phase Separation of Proteins with High-Resolution and High-Column Efficiency

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Abstract: A low-sized, uniform and polymer-based high-performance liquid chromatography (HPLC) packing material capable of making reversed-phase separation of proteins with high resolution and with high column efficiency was developed. By a multistage-swelling and polymerization protocol, 5 μm-uniform-porous poly(styrene-co-divinylbenzene) particles with relatively larger pores particularly suitable for protein separation were synthesized by starting from a low-sized seed latex with high average molecular weight and by using a diluent phase comprised of dibutylphthalate and toluene. By the use of synthesized beads as packing material in HPLC, high-resolution liquid chromatograms were obtained in the gradient separation of selected proteins (i.e., ribonuclease-A, lysozyme, cytochrome C, and albumin). In the chromatographic runs, the flow rate of the mobile phase was increased fourfold by preserving the resolution power of the column material under gradient conditions. The theoretical plate numbers (TPN) up to 12.500 plates/m were observed by using cytochrome C as the analyte. TPN values determined by the proteins were significantly higher relative to

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the similar uniform packing materials larger in size (i.e., 7.5–10 μm) obtained by different polymerization methods.

Keywords: HPLC, reversed-phase chromatography, chromatographic packing, protein separation, divinylbenzene, uniform particles, porous particles

INTRODUCTION

There has been considerable research effort on the separation of proteins by different chromatographic methods. In preparative scale, various affinity chromatography applications were developed for the separation of proteins. Dye carrying stationary phases were used for the isolation of different proteins including albumin, lactate hydrogenase, alcohol dehydrogenase (1–5). The interaction of poly(vinyl alcohol) coated macroporous poly(styrene-divinylbenzene) particles with albumin was investigated by Leonard et al. (3). Dye carrying and polyvinyl alcohol modified macroporous poly(S-DVB) particles were used for the purification of different proteins like HSA and lysozyme (4).

High-performance liquid chromatography (HPLC) and capillary electrochromatography (CEC) are efficient methods in the separation of proteins. Today, uniform-porous beads are known as a relatively new and an important family of HPLC packing materials. Ugestad et al. developed "multistage activated swelling method" for the production of uniform-porous particles in the range of 1–20 μm (6). Until today, various methods were proposed by different researchers for the production of uniform-porous particles suitable as packing material in HPLC (7–16). By starting from the polystyrene seed latex prepared by dispersion polymerization, Ogino et al. proposed a single-stage swelling and polymerization method for the production of uniform poly(styrene-divinylbenzene) and oligo(ethyleneglycol dimethacrylate) particles in the size range of 4–10 μm (16).

The separation media in the form of continuous beds were developed for the reversed phase chromatography and anion exchange chromatography of proteins in the capillary mode (17, 18). The separation of proteins in monolithic media was also studied by different researchers (19, 20). Uniform poly(glycidyl methacrylate-ethylene dimethacrylate) beads 3 μm in size carrying propyl sulfonic acid moieties were used as packing material for the separation of proteins by CEC operated in ion-exchange mode (21). The polydisperse, spherical, and porous particles obtained by suspension polymerization with different surface chemistries were also tried as packing material in HPLC for the separation of proteins (22, 23). Reversed-phase HPLC of peptides of pepsin hydrolyzed by the use of various forms of immobilized α-chymotrypsin onto the uniform latex beads was achieved (24) Gong et al. proposed uniform-macroporous poly(glycidyl methacrylate-ethylene dimethacrylate) based beads for

protein separation both by hydrophobic interaction and ion-exchange chromatography (25, 26).

We also proposed a multistage polymerization protocol, the so-called "modified seeded polymerization" for the production of uniform-porous particles in the range of 7.5–15 μm. The method was applied on the production of uniform porous particles carrying different functional groups like hydroxyl, carboxyl, epoxypropyl, and boronic acid (27–30). The uniform porous poly(styrene-co-divinylbenzene) particles 7.5 μm in size produced by our protocol were successfully tried as packing material in HPLC (31, 32).

In the HPLC applications, the separation efficiency increases with decreasing particle size of the packing material. The uniform-porous particles lower than 5 μm are particularly suitable for the chromatographic separations in micro or semimicro HPLC with high column efficiency and high resolution. Hence it is possible to make the chromatographic analysis by reducing the volume of mobile phase and in a shorter time. No study could be found in the literature on the high-resolution reversed phase separation of proteins by using a polymer-based HPLC column including uniform particles smaller than 5 μm.

For this purpose, our polymerization protocol, the so-called "modified seeded polymerization" was changed for reducing the size of porous particles and for obtaining a pore structure suitable for the separation of large molecules like proteins. Hence, a low-sized, uniform and polymer-based HPLC packing material capable of making the reversed-phase separation of proteins with high resolution and with high column efficiency was developed. We wish to report the chromatographic performance of this material comparatively with the packing materials larger in size.

EXPERIMENTAL

Materials

Styrene (Yarpet AS, Turkey) was distilled under vacuum and stored in the refrigerator until use. Sodium lauryl sulfate (SLS, Sigma Chem. Co., St. Louis) was used in the preparation of aqueous emulsion medium for the swelling of PS seed particles. Toluene and dibutyl phthalate (DBP) (HPLC grade, Aldrich Chem. Co., Milwaukee, WI) were the components of the diluent mixture used in the particle synthesis. Divinylbenzene (55% para and meta-divinylbenzene isomers, Aldrich Chem. Co.) was treated with 5% w/w NaOH solution for the removal of inhibitor. Benzoyl peroxide (BPO, Aldrich Chem. Co.) was the initiator in the repolymerization of monomer phase within the swollen seed particles. Acetonitrile (AcN, HPLC grade) was supplied from Aldrich Chem. Co. The proteins, 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), Cytochrome-C (Cat No: C-2037, MW: 12.327) were purchased from Sigma Chem. Co. Trifluoroacetic acid (TFA) was supplied from Aldrich Chem. Co.

Synthesis of Uniform-Porous Particles

Two types of porous uniform poly(styrene-co-divinylbenzene) particles were prepared by the use of polystyrene (PS) seed latexes with different sizes. The detailed synthesis protocols of both types of particles by "modified seeded polymerization" were given elsewhere (33). The first one, small porous particles (d_p: 5.2 μm) were prepared by starting from a PS seed latex 2.1 μm in size obtained by dispersion polymerization. Typically, the seed particles were swollen in an aqueous emulsion medium by a diluent mixture comprised of toluene and DBP with equal volumes and then by a monomer mixture including styrene, divinylbenzene and benzoyl peroxide. In the last stage, the monomer phase was polymerized within the swollen seed particles. Large porous poly(styrene-co-divinylbenzene) particles (d_p: 7.8 μm) were obtained by starting from another PS seed latex 4.4 μm in size. A preparation protocol similar to that described previously was applied except pure DBP was utilized as the diluent instead of toluene-DBP mixture. Following the synthesis, the particles were washed with ethanol and then extracted by tetrahydrofuran (THF). The size and surface structure of the particles were investigated by scanning electron microscopy. The porosity characteristics of the particles were determined by a SEC study according to the method described by Blondeau's group (34).

Chromatographic Study

The particles were slurry packed into 150 mm × 4.6 mm I.D. stainless steel HPLC columns. The chromatography was carried out using a Shimadzu gradient liquid chromatograph (LC-10 ADVP) equipped with a SPD-10 AVVP UV detector. In the chromatographic experiments performed with gradient mode, bovine serum albumin, lysozyme, ribonuclease A, and cytochrome-C were used as the protein standards. The separation of these proteins was studied under AcN/water linear gradient at room temperature. The chromatograms were obtained at different flow rates ranging between 0.25 and 2.0 mL/min at 280 nm. Triflouroacetic acid (TFA, Aldrich. Chem. Co.) was included in both acetonitrile and water phases at a concentration of 0.15% (v/v).

The peak-resolution was determined 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 of 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, plates/meter) was calculated from the peaks of cytochrome C in a water/acetonitrile mixture (50:50 or 30:70) at different flow rates between 0.25–1.0 mL/min based on Eq. (2) where L is the column length, t_r and t_w are the retention time and the peak-width at half height, respectively.

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

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

RESULTS AND DISCUSSION

Characterization of Particles

The SEM photographs of two types of uniform-porous particles 5.2 and 7.8 μm in size are given in Fig. 1. The size and porosity properties are

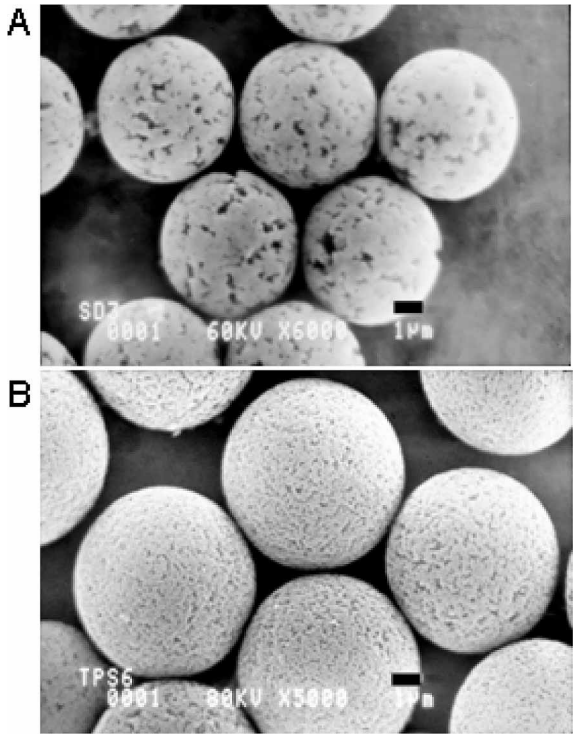

Figure 1. The SEM photographs of porous particles, Particle type: (A) SD3, (B) SD5 (Magnification: 6000X for (A) and 5000X for (B)). The bar on each photograph corresponds 1 μm.

summarized in Table 1. The coefficient of variation (CV) values indicated that both types of particles were produced with reasonably narrow size distribution. As seen both in Fig. 1 and Table 1, the particles 5.2 μm in size (i.e., encoded SD3) had a porous structure with a larger average size with respect to the particles encoded as SD5. In other words, the presence of macropores was more clearly observed on the surface of SD3 while SD5 had a typical sponge-like porosity. Although the pore volumes of both materials are approximately equal, the specific surface area of SD5 was slightly higher probably due to its sponge-like porosity. As a typical HPLC packing material with relatively larger particle size, SD5 was used as reference for the evaluation of the chromatographic performance of the particles with relatively smaller size (i.e., 5.2 μm) in the reversed-phase separation of proteins.

The weight average molecular weights (M_w) of the seed latexes used for the synthesis of small and large porous particles were 1.02×10^5 and 2.16×10^4 (33). The use of seed latex with higher MW usually involve higher pore size in the resulting poly(styrene-co-divinylbenzene) particles based on the pore-formation mechanism proposed elsewhere (7, 8, 31, 32). It is also evident that the selection of seed latex with smaller size involves the synthesis of smaller poly(styrene-co-divinylbenzene) particles under similar production conditions. As seen here, the porous poly(styrene-co-divinylbenzene) particles with relatively larger average pore size (i.e., SD3) could be achieved by the use of a sufficiently small seed latex with relatively larger molecular weight.

Chromatographic Evaluation

In the first stage of the chromatographic runs, the reversed-phase separation of proteins was investigated under acetonitrile (AcN)-water gradient. In these runs, the sample was a protein mixture containing ribonuclease-A (10 mg/mL), lysozyme (5 mg/mL), cytochrome-C (5 mg/mL), and albumin (25 mg/mL). The chromatographic conditions are described in Table 2. As seen here, the linear gradients with three different slopes were applied by starting with 30%v/v AcN concentration. The stainless steel columns (150 × 4.6 mm i.d.) packed with SD3 and SD5 were used with a UV detector operated at 280 nm. The resolutions calculated based on the liquid

Table 1. The size and porosity properties of porous particles (33)

Code	Particle size (μm)	CV(%)	Average pore size (nm)	Pore volume (mL/g)	Porosity (% v/v)	Specific surface area (m^2/g)
SD3	5.2	3.4	95	1.02	56.4	43.3
SD5	7.8	2.5	62	1.05	55.9	67.8

Table 2. The conditions for linear gradients used for the separation of proteins by reversed-phase chromatography on the columns packed with small and large porous particles. Conditions: Column: 150 × 4.6 mm i.d; Mobile phase; (A): 5% water in acetonitrile + 0.15% TFA, (B): water + 0.15% TFA, Flow rate: 0.75 mL/min.; UV detection at 280 nm

Initial A (%)	Final A (%)	Time (min)	Slope (percent/min)
30	60	30	1.0
30	60	20	1.5
30	70	20	2.0

chromatograms obtained with different slopes are listed in Table 3. As known, the resolution of 1.5 is accepted as a threshold value for satisfactory resolution between two successive peaks in a liquid chromatogram. As seen in Table 3, the proteins could be separated in both columns by applying linear gradients with slopes varying between 1 and 2. For a linear gradient applied with a certain slope, the resolutions obtained with SD3 were significantly higher with respect to those of SD5 under identical conditions. Additionally, an appreciable decrease was observed in all resolutions with the increasing slope of the linear gradient in the presence of SD5. However, there was no significant decrease in the resolutions obtained with SD3 in the case of a sharper gradient. These findings indicated that SD3 exhibited better chromatographic performance in the reversed-phase separation of the selected proteins with all gradients. The chromatographic behaviour of SD3 should be probably explained by the lower particle size and more suitable pore structure

Table 3. The resolutions calculated based on the liquid chromatograms obtained by the linear gradients with different slopes. The chromatographic conditions are described in Table 2. Order of elution: Peak 1. Ribonuclease-A, Peak 2. Cytochrome-C, Peak 3. Lysozyme, Peak 4. Albumin

Slope (percent/min)	R(2/1)	R(3/2)	R(4/3)
Column: SD3			
1.0	14.82	3.40	5.30
1.5	18.42	3.39	5.51
2.0	18.83	3.28	4.02
Column: SD5			
1.0	12.65	3.36	4.24
1.5	12.26	2.82	3.41
2.0	11.30	1.83	2.20

allowing the diffusion of large molecules like proteins. Note that the resolutions obtained with SD3 were significantly higher with respect to those obtained in the protein separations with different chromatographic methods including aqueous size exclusion, ion exchange, and hydrophobic interaction modes operated with different packing materials based on uniform-porous polymer particles produced by different methods (6, 10, 16, 25, 26).

The effect of mobile phase flow rate on the chromatographic behavior of the column materials was investigated under constant gradient conditions. For this purpose, the slope of the gradient was fixed to 1.5 percent/min for both columns based on the behavior observed in the previous set. The other chromatographic conditions were the same with those of the previous set. The chromatograms obtained with different mobile phase flow rates are given in Fig. 2 for both columns. The resolutions calculated for the chromatograms given in Fig. 2 are presented in Table 4. As expected, the resolutions obtained with SD3 were significantly higher than those of SD5.

The effects of flow rate on the theoretical plate number (TPN) are given in Fig. 3. For both columns, TPN exhibited a maximum at 0.25 mL/min and then slightly decreased with the increasing flow rate. Note that TPN values for both columns were determined by using cytochrome-C as the analyte. As seen here, TPN values up to 12.500 plates/m could be achieved with the column

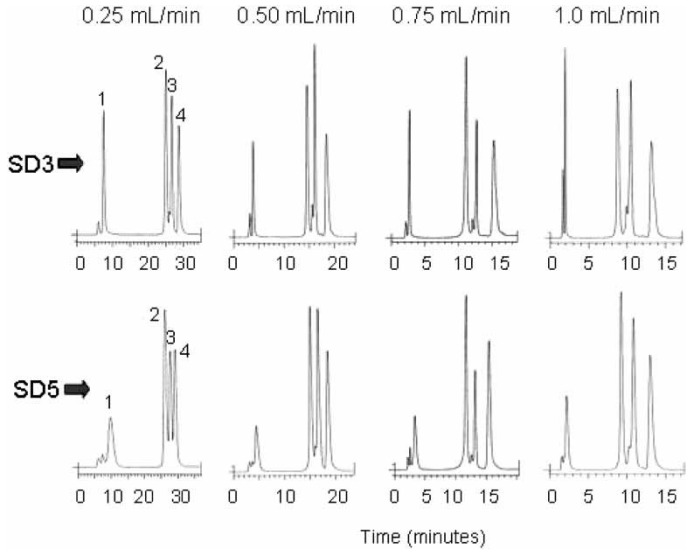

Figure 2. The liquid chromatograms obtained under gradient conditions with different mobile phase flow rates for the reversed phase separation of proteins in the columns packed with small and large particles, Chromatographic conditions, Column: 150 × 4.6 mm i.d., Mobile phase; (A): 5% water in AcN + 0.15% TFA, (B): water + 0.15% TFA, Flow rate:0.75 mL/min, UV detector, 280 nm. Order of elution: Peak 1. Ribonuclease-A, Peak 2. Cytochrome-C, Peak 3. Lysozyme, Peak 4. Albumin.

Table 4. The resolutions calculated based on the liquid chromatograms obtained by the linear gradients with different mobile phase flow rates. Order of elution: Peak 1. Ribonuclease-A, Peak 2. Cytochrome-C, Peak 3. Lysozyme, Peak 4. Albumin

Flow rate (mL/min)	R(2/1)	R(3/2)	R(4/3)
Column: SD3			
0.25	20.25	1.85	2.10
0.50	18.38	2.58	3.23
0.75	18.42	3.39	5.51
1.00	16.41	3.04	6.04
Column: SD5			
0.25	6.88	1.07	0.90
0.50	10.20	1.93	2.02
0.75	12.26	2.82	3.41
1.00	11.54	2.49	2.76
1.50	11.26	2.58	2.99
2.00	12.07	2.68	3.55

including the particles 5.2 μm in size. However, the reference column packed with the particles 7.8 μm in size gave TPN values varying between only 3000–4000 plates/m. The TPN values determined for the column packed with the particles 5.2 μm in size were also higher with respect to those

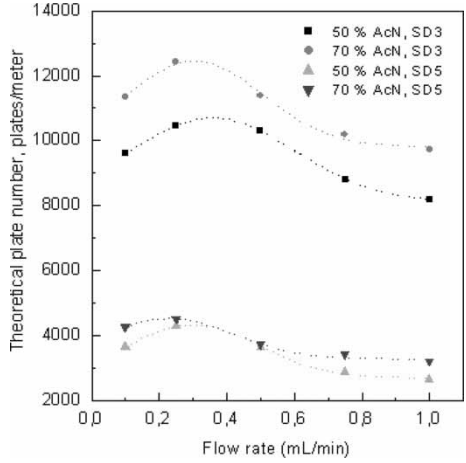

Figure 3. The effect of mobile phase flow rate on the theoretical plate number of the columns packed with relatively small and large particles, Conditions: Column dimensions: 150 × 4.6 mm i.d., Mobile phase, AcN-water (70:30 or 50:50 v/v) with 0.15% TFA, Gradient slope:1.5 percent/min., UV detection at 280 nm, Analyte: cytochrome-C.

obtained with the uniform-porous polymer particles used in different chromatographic modes (6, 10, 16, 25, 26).

The effect of mobile phase flow rate on the back pressure is given in Fig. 4 for the columns packed with SD3 and SD5. As seen here, the back-pressure vs. flow rate plots were sketched for three different AcN concentrations. As seen here, SD3 provided higher back-pressure with respect to SD5 at constant flow rate. Of course, the smaller average size should be the reason of the higher back-pressures obtained with SD3. Based on this behavior, higher mobile phase flow rates could be used for the reversed-phase separation of proteins under AcN-water gradient by using the columns packed with SD5. The chromatograms obtained with higher flow rates by using the column packed with SD5 are given in Fig. 5. As seen by the comparison of Figs. 2 and 5, the main advantage of higher flow rate is of course to complete the chromatographic analysis in a shorter time. The resolution values calculated based on these chromatograms are presented in Table 4. It should be noted that satisfactory high resolutions were also obtained, although the chromatographic separation was completed in reasonably shorter times by using SD5 as the packing material.

CONCLUSION

A uniform-porous polymer particle-based packing material was developed for the reversed-phase separation of proteins. In order to achieve high column efficiency and high resolution in the chromatographic separation, relatively small, uniform-porous polymer particles with large pore suitable for the separation of

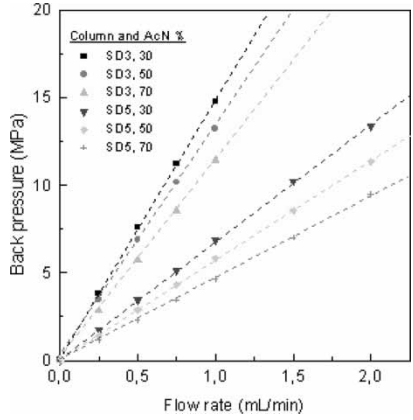

Figure 4. The variation of back pressure with the mobile phase flow rate for the columns packed with small and large particles. Conditions: Column dimensions: 150 × 4.6 mm i.d., Mobile phase: AcN-water.

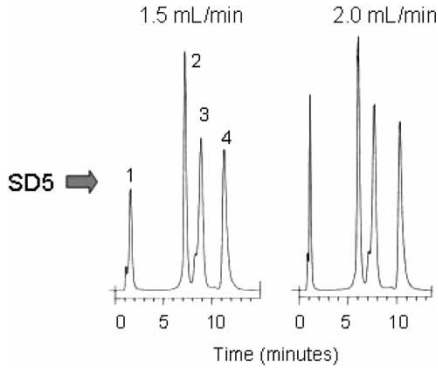

Figure 5. The liquid chromatograms obtained under gradient conditions with different mobile phase flow rates for the reversed phase separation of proteins in the column packed with large particles (SD5), Chromatographic conditions, Column: 150 × 4.6 mm i.d., Mobile phase; (A): 5% water in acetonitrile + 0.15% TFA, (B): water + 0.15% TFA, Gradient slope:1.5 percent/min., Flow rate: 0.75 mL/min, UV detector, 280 nm. Order of elution: Peak 1. Ribonuclease-A, Peak 2. Cytochrome-C, Peak 3. Lysozyme, Peak 4. Albumin.

proteins were obtained by modified seeded polymerization. The theoretical plate numbers (TPN) up to 12.500 plates/m could be achieved by using cytochrome C as the analyte. TPN values determined by the proteins were approximately twofold higher relative to the similar uniform packing materials with relatively larger size (i.e., 7.5–10 μm) obtained by different polymerization methods or by modified seeded polymerization. The flow rate of the mobile phase was increased fourfold by preserving the resolution power of the column material in gradient mode. As a conclusion, the proposed material exhibited a superior chromatographic performance in the reversed-phase separation of proteins with respect to the polymer-based HPLC packing materials.

REFERENCES

1. Lowe, C.R., Glad, M., Larsson, P.O., Ohlson, S., Small, D.A.P., Atkinson, T., and Mossbach, K. (1981) High-performance liquid affinity chromatography of proteins on cibacron blue F3G-A bonded silica. *Journal of Chromatography*, 215 (2): 303–316.
2. Lee, W.C. (1997) Protein separation using non-porous sorbents. *Journal of Chromatography B, Biomedical Applications*, 699 (1): 29–45.
3. Leonard, M., Fournier, C., and Dellacherie, E. (1995) Polyvinyl alcohol-coated macroporous polystyrene particles as stationary phases for the chromatography of proteins. *Journal of Chromatography B, Biomedical Applications*, 664 (1): 39–46.

4. Nash, D.C., McCreath, G.E., and Chase, H.A. (1997) Modification of polystyrenic matrices for the purification of proteins effect of the adsorption of poly(vinyl alcohol) on the characteristics of poly(styrene-divinylbenzene) beads for use in affinity chromatography. *Journal of Chromatography A*, 758 (1): 53–64.
5. Camli, S.T., Senel, S., and Tuncel, A. (1999) Cibacron blue F3G-A-attached uniform and macroporous poly(styrene-co-divinylbenzene) particles for specific albumin adsorption. *Journal of Biomaterials Science Polymer Edition*, 10 (8): 875–889.
6. Ellingsen, T., Aune, O., Ugelstad, J., and Hagen, S. (1990) Monosized stationary phases for chromatography. *Journal of Chromatography*, 535 (1): 147–161.
7. Cheng, C.M., Micale, F.J., Vanderhoff, J.W., and El-Aasser, M.S. (1992) Synthesis and characterization of monodisperse porous polymer particles. *Journal of Polymer Science: Part A: Polymer Chemistry*, 30 (2): 235–244.
8. Cheng, C.M., Vanderhoff, J.W., and El-Aasser, M.S. (1992) Monodisperse porous polymer particles: Formation of the porous structure. *Journal of Polymer Science: Part A: Polymer Chemistry*, 30 (2): 245–256.
9. Galia, M., Svec, F., and Frechet, J.M.J. (1994) Monodisperse pollymer beads as packing material for high-performance liquid chromatography: Effect of divinylbenzene content on the porous and chromatographic properties of poly(styrene-co-divinylbenzene) beads prepared in presence of linear polystyrene as a porogen. *Journal of Polymer Science: Part A: Polymer Chemistry*, 32 (14): 2169–2175.
10. Wang, Q.C., Svec, F., and Frechet, J.M.J. (1994) Fine control of the porous structure and chromatographic properties of monodisperse macroporous poly (styrene-co-divinyl benzene) beads using polymer porogens. *Journal of Polymer Science: Part A: Polymer Chemistry*, 32 (15): 2577–2588.
11. Tuncel, A., Tuncel, M., and Salih, B. (1999) Electron microscopic observation of uniform porous particles. I. Effect of seed latex type and diluent. *Journal of Applied Polymer Science*, 71 (14): 2271–2290.
12. Tuncel, A. (1999) Electron microscopic observation of uniform macroporous particles. II. Effect of DVB concentration. *Journal of Applied Polymer Science*, 71 (14): 2291–1302.
13. Tuncel, A., Unsal, E., Camli, S.T., and Senel, S. (2003) Polymer beads in biomedical chromatography. In *Colloidal Biomolecules, Biomaterials, and Biomedical Applications*; Elaissari, A., ed.; Marcel Dekker: New York, 161–187.
14. Li, W.H. and Stöver, H.D.H. (2000) Monodisperse cross-linked core-shell polymer microspheres by precipitation polymerization. *Macromolecules*, 33 (12): 4354–4360.
15. Ogino, K., Sato, H., Tsuchiya, K., Suzuki, H., and Moriguchi, S. (1995) Synthesis of monodisperse macroreticular styrene-divinylbenzene gel particles by a single-step swelling and polymerization method. *Journal of Chromatography A*, 699 (1–2): 59–66.
16. Ogino, K., Sato, H., Aihara, Y., Suzuki, H., and Moriguchi, S. (1995) Preparation and characterization of monodisperse oligo(ethylene glycol) dimethacrylate polymer beads for aqueous high-performance liquid chromatography. *Journal of Chromatography A*, 699 (1–2): 67–72.
17. Ericson, C. and Hjerten, S. (1999) Reversed-phase electrochromatography of proteins on modified continuous beds using normal-flow and counterflow gradients. *Theoretical and Practical Considerations. Analytical Chemistry*, 71 (8): 1621–1627.

18. Li, Y.M., Liao, J.L., Zhang, R., Henriksson, H., and Hjerten, S. (1999) Continuous beds for microchromatography: Chromatofocusing and anion exchange chromatography. *Analytical Biochemistry*, 267 (1): 121–124.
19. Podgornik, A., Barut, M., Jaksa, S., Sancar, J., and Strancar, A. (2002) Application of very short monolithic columns for separation of low and high molecular mass substances. *Journal of Liquid Chromatography & Related Technologies*, 25 (20): 3099–3116.
20. Josic, D. and Buchacher, A. (2001) Application of monoliths as supports for affinity chromatography and fast enzymatic conversion. *Journal of Biochemical and Biophysical Methods*, 49 (1–3): 153–174.
21. Zhang, S., Zhang, J., and Horvath, C. (2002) Capillary electrochromatography of proteins with polymer-based strong-cation-exchanger microspheres. *Journal of Chromatography A*, 965 (1–2): 83–92.
22. Paredes, B., Suarez, E., Rendueles, M., Villa-Garcia, M.A., and Diaz, J.M. (2001) Ammonia-functionalized particulate poly(glycidyl methacrylate-co-ethylene dimethacrylate)-based polymers as stationary phase for protein retention. *Journal of Chemical Technology and Biotechnology*, 76 (11): 1171–1178.
23. Shi, Y. and Sun, Y. (2003) Fabrication and characterization of a novel biporous spherical adsorbent for protein chromatography. *Chromatographia*, 57 (1–2): 29–35.
24. Vankova, H., Kucerova, Z., and Turkova, J. (2001) Reversed-phase high-performance liquid chromatography of peptides of porcine pepsin prepared by the use of various forms of immobilized alpha-chymotrypsin. *Journal of Chromatography B*, 735 (1): 37–43.
25. Gong, B., Ke, C., and Geng, X. (2003) Preparation of weak cation exchange packings based on monodisperse poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads and their chromatographic properties. *Analytical and Bioanalytical Chemistry*, 375 (6): 769–774.
26. Gong, B., Wang, L., Wang, C., and Geng, X. (2004) Preparation of hydrophobic interaction chromatographic packings based on monodisperse poly(glycidylmethacrylate-*co*-ethylenedimethacrylate) beads and their application. *Journal of Chromatography A*, 1022 (1–2): 33–39.
27. Camli, T., Tuncel, M., Senel, S., and Tuncel, A. (2002) Functional, uniform and macroporous latex particles: Preparation, electron microscopic characterization, and nonspecific protein adsorption properties. *Journal of Applied Polymer Science*, 84 (2): 414–429.
28. Tuncel, A., Tuncel, M., Ergun, B., Alagöz, C., and Bahar, T. (2002) Carboxyl carrying-large uniform latex particles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 197 (1–3): 79–94.
29. Tuncel, A., Tuncel, M., Cicek, H., and Fidanboy, O. (2001) 2-Hydroxyethylmethacrylate carrying uniform porous particles: Preparation and electron microscopy. *Polymer International*, 51 (1): 75–84.
30. Senel, S., Çamli, S.T., and Tuncel, A. (2002) Nucleotide adsorption-desorption behaviour of boronic acid functionalized uniform-porous particles. *Journal of Chromatography B*, 769 (2): 283–295.
31. Unsal, E., Camli, S.T., Senel, S., and Tuncel, A. (2004) Chromatographic performance of monodisperse-macroporous particles produced by "modified seeded polymerization" I: Effect of monomer/seed latex ratio. *Journal of Applied Polymer Science*, 92 (1): 607–618.

32. Camli, S.T., Unsal, E., Senel, S., and Tuncel, A. (2004) Chromatographic performance of monodisperse macroporous particles produced by modified seeded polymerization. II. Effect of diluent/seed-latex ratio. *Journal of Applied Polymer Science*, 92 (6): 3685–3696.
33. Unsal, E., Camli, S.T., Elmas, B., Tuncel, M., Senel, S., and Tuncel, A. (2004) Monodisperse-porous poly(styrene-divinyl benzene) beads providing high column efficiency in reversed phase HPLC. *Journal of Applied Polymer Science*, *In Press*.
34. Ferreira, A., Bigan, M., and Blondeau, D. (2003) Optimization of a polymeric HPLC phase: Poly(glycidyl methacrylate-co-ethylene dimethacrylate) influence of the polymerization conditions on the pore structure of macroporous beads. *Reactive and Functional Polymers*, 56 (2): 123–136.