ELSEVIER

Journal of Chromatography A, 924 (2001) 187–195

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Photopolymerized sol–gel frits for packed columns in capillary electrochromatography

Masaru Kato[1], Maria T. Dulay, Bryson D. Bennett, Joselito P. Quirino, Richard N. Zare*

Department of Chemistry, Stanford University, Stanford, CA 94305-5080, USA

Abstract

Porous sol–gel frits are fabricated in a capillary column by filling it with a solution of 3-(trimethoxysilyl)propyl methacrylate, hydrochloric acid, water, toluene (porogen), and a photoinitiator (Irgacure 1800) and exposing it to UV light at 365 nm for 5 min. The separation column (30 cm×75 μm I.D.) contains between the inlet and outlet frits a 15-cm packed segment filled with 5-μm silica particles modified with the chiral compound (*S*)-*N*-3,5-dinitrobenzoyl-1-naphthylglycine. A detection window (1 mm long) is placed immediately after the outlet frit. To demonstrate the performance of this chiral separation column, mixtures of 16 different amino acids (three of which are not naturally occurring) derivatized with the fluorogenic reagent 4-fluoro-7-nitro-2,1,3-benzoxadiazole were separated by capillary chromatography. The enantiomeric separation of the column results in a resolution ranging from 1.21 to 8.29, and a plate height ranging from 8.7 to 39 μm.

Keywords: Sol–gel; Electrochromatography; Enantiomer separation; Amino acids

1. Introduction

Capillary electrochromatography (CEC) is a technique that combines the high separation efficiency of capillary electrophoresis (CE) with the versatile separation mode and loading capacity of high-performance liquid chromatography (HPLC) [1,2]. CEC has become a promising technique for the separation of analytes based on both electrophoresis and partitioning between a mobile and a stationary phase. Hence, CEC can separate both charged and uncharged compounds with high resolution and efficiency in the same run [3–7]. CEC has been used in many applications, such as for environmental, pharmaceutical, and biological samples, and many review articles have reported on this topic [8–12].

Capillary columns for CEC divide into three types: (1) surface-functionalized open tubular columns [13–16], (2) monolithic capillary columns [17–24], and (3) packed capillary columns with retaining frits [3–7,25–27]. While open tubular columns are easy to fabricate, they suffer from low retention and low sample capacities (with the typical capillary opening being about 50 μm). Monolithic columns have gained interest because they offer fritless columns that can be easily and reproducibly fabricated. Reversed-phase and chiral separations are accomplished using monolithic columns [22,28]. These monolithic structures, however, are often restricted to the use of monomers, most of which are not porous after polymerization. Presently, packed columns are the

*Corresponding author. Tel.: +1-650-7233-062; fax: +1-650-7250-259.

E-mail address: zare@stanford.edu (R.N. Zare).

[1]Present address: Department of Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka, Shizuoka 422-8526, Japan.

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00772-5

most widely used as CEC columns. The packed columns contain the packing particles for HPLC or specially designed packing particles for CEC. A wide variety of compounds, such as environmental, biological, and chiral samples, are separated on packed columns [3–6,25]. The retaining frits, however, remain the most problematic step in the fabrication of packed columns. Presently, difficulties remain in the preparation of frits with reproducible porosities and lengths [29–33].

Recently Chen et al. [34] have reported on the photochemical fabrication of frits made of glycidyl methacrylate and trimethylolpropane trimethacrylate Through photochemical means, it becomes easy to control the frit length and position in the capillary, because the frit is fabricated only where light was irradiated. This photopolymerization method offers two advantages in the generation of porous frits in a capillary column. The frit is strong enough to withstand a short exposure of high pressure (>6000 p.s.i.; 1 p.s.i.=6894.76 Pa). By changing the composition of the monomer solution, the porous properties of the frits were changed [34]. We report here a variation in which we use photopolymerization of sol–gel to create the frit.

2. Experimental

2.1. Apparatus

All separations were performed on a Beckman P/ACE 5000 capillary electrophoresis system (Fullerton, CA, USA). The instrument was equipped with an air-cooled 488-nm argon ion laser. Fused-silica capillaries (75 μm I.D.×365 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). An XL-1500 UV crosslinker (Spectronics, Westbury, NY, USA) which has six 15 W fluorescent blacklight tubes, producing UV light of predominantly 365 nm wavelength, was used for irradiation of the monomer solutions. Scanning electron microscopy (SEM) analyses were performed on a scanning electron microscope (Philips SEM 505, Eindhoven, The Netherlands).

2.2. Reagents

The 5-μm spherical chiral particles modified with (*S*)-*N*-3,5-dinitrobenzoyl-1-naphthylglycine were donated from the Graduate School of Pharmaceutical Sciences, University of Tokyo (Tokyo, Japan) and Sumika Chemical Analysis Service (Osaka, Japan) (Fig. 1). D- and L-Amino acids, D- and L-non-protein amino acids (NPAAs), 3-(trimethoxysilyl)propyl methacrylate and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) were purchased from Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA) or Fluka (Ronkonkoma, NY, USA) and were used as received. Irgacure 1800 was from Ciba (Tarrytown, NY, USA).

Doubly distilled water was used in the preparation of all samples and buffers. HPLC-grade acetonitrile was purchased from Aldrich and used without further purification.

2.3. Preparation of photopolymerized sol–gel frit column

The sol–gel solution was made up of 750 μl of 3-(trimethoxysilyl)propyl methacrylate, 22.5 μl of 0.12 *M* hydrochloric acid and 225 μl of water, and was stirred for 30 min in the dark at room temperature. A 170-μl volume of toluene was added to 30 μl of the sol–gel solution and stirred for 30 min at room temperature. An 8.9-mg amount of Irgacure 1800 was added to the toluene mixture and stirred for 1 h at room temperature. This procedure forms what we refer to as solution A.

The outlet frit was prepared first. About a 3-mm section of the polyimide coating about 10 cm from the end of a 30-cm long capillary was removed with a razor. The capillary was then filled with solution A

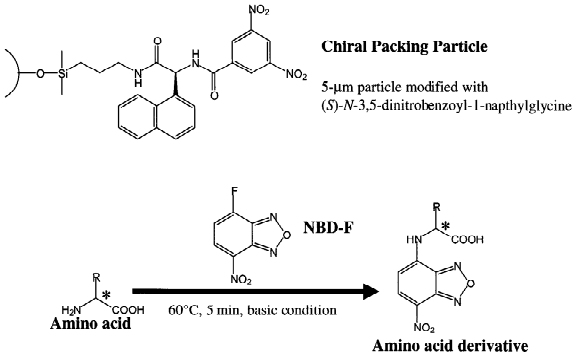

Fig. 1. Chemical structure of packing particle and derivatization scheme of amino acid with fluorogenic reagent.

using a syringe. Before the capillary was exposed to UV light for 5 min, both ends of the capillary were sealed with parafilm. The presence of a frit was confirmed by inspection at 100× magnification. The monolithic material has an opaque appearance and is very porous. The capillary was rinsed with ethanol by pressure from a syringe to remove the unreacted solution. A 15-cm packed chiral section was prepared in the capillary by introducing a sonicated (for 5 min) slurry of 10 mg of chiral particles into the capillary column with a syringe and a hand-held vise. Lastly, the inlet frit was prepared in the column in the same manner as the outlet frit. A 3-mm section of the polyimide coating about 25 cm from the outlet of the capillary and 15 cm from the outlet frit was removed. Solution A was introduced into the capillary with a syringe pressurized with a hand-held vise. The resulting frit is located immediately at the end of the packed section. A detection window was created immediately after the packed section at the outlet by using hot sulfuric acid (>100°C). The column was preconditioned with running buffer that had been degassed by sonication (by pressurizing the column inlet to approx. 200 p.s.i. with a hand-held vise). Next, the column was further conditioned in the CE instrument by electrokinetically driving the buffer mobile phase through the capillary at an applied voltage of 15 kV until a stable baseline was achieved. This procedure typically takes 2–3 h to complete.

2.4. Derivatization of amino acids

A 10-μl volume of each 5 m*M* amino acid or NPAA in 0.2 *M* borate buffer (pH 8.0) and 10 μl of 5 m*M* NBD-F in acetonitrile were mixed and heated at 60°C for 5 min (Fig. 1). After addition of 20 μl of running buffer, the mixture was electrokinetically injected into the capillary at 10 kV for 5 s.

2.5. Separation

A capillary column with a 15-cm chiral packed section was used for the separation of amino acids. The derivatized amino acid sample was injected into the column electrokinetically (0.33 kV/cm) at a temperature of 20°C. Applied voltages during the separations are mainly 0.83 kV/cm or 0.50 kV/cm. The elution time of an unretained compound is taken to be the time from injection to the occurrence of the first solvent disturbance peak. The velocity of the first disturbance peak is 1.28 mm/s, when 0.83 kV/cm is applied through the column. The analytes were observed by monitoring their fluorescence intensities (excitation wavelength is 488 nm with a band pass filter of 520 nm for emission). The efficiency of an enantiomeric separation is measured by the value of the resolution factor, which is defined as:

$$\text{Resolution} = 2(t_A - t_B)/(W_A + W_B)$$

where t_A is the retention time of a more retained enantiomer (A), t_B is the retention time of a less retained enantiomer (B), and W_A and W_B are peak widths of species A and B.

2.6. SEM analysis

A packed capillary was sectioned into 5-mm segments. These segments were sputtered with gold for SEM analyses.

3. Results and discussion

3.1. SEM study

The packed capillaries used in the experiments described in this paper can be thought of as having three sections: (1) the outlet frit, (2) the packed section, and (3) the inlet frit. As seen in Fig. 2, the outlet frit appears to be made of a network of interconnecting 1-μm diameter spherical structures. There are no particles embedded within the outlet frit. There are 3-μm channels (dark areas) seen throughout the sol–gel network. These channels allow passage of ions and liquid, but prevent the escape of chiral particles. The inlet frit, however, does contain some embedded chiral particles (Fig. 3). This is a result of the particles mixing with solution A as it entered the packed section of the capillary prior to irradiation. The interconnecting spherical structures that comprise the outlet frit is no longer apparent. Instead, the SEM micrograph (Fig. 3) shows some amorphous structure that covers and binds the chiral particles to form the inlet frit. The structural differences observed between the two frits in the presence and absence of particles is similar to that reported in other papers [34,35]. Fig. 4 is a

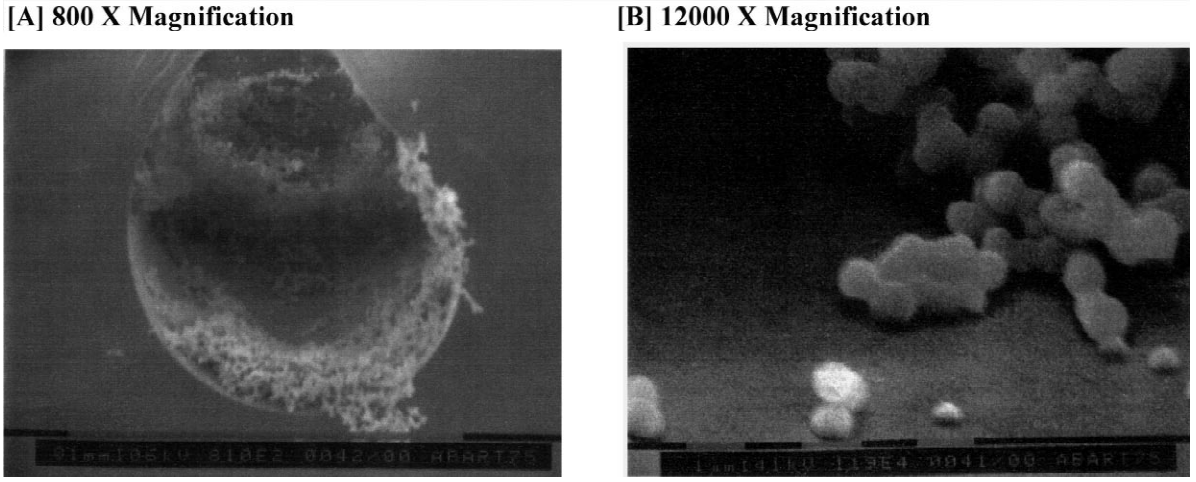

Fig. 2. Scanning electron micrographs of the outlet frit of the CEC capillary column.

micrograph of a section of the packed segment of the capillary. The sol–gel material did not form in any part of the packed segment because the polyimide coating blocked the UV light from entering this section of the capillary during the photopolymerization of the frits. Therefore, the packed segment is only made up of chiral particles that are held in place by the outlet and inlet frits.

3.2. Chiral separation

The performance of the packed chiral columns was studied by separating fluorescently derivatized amino acids. The results were then compared to previously reported amino acid separations done on monolithic columns using a sol–gel material to embed the same chiral particles [28]. In the previous report, mixtures of 13 derivatized amino acids and three NPAAs were separated on a chiral particle-loaded monolithic column using a separation solution of 5 m*M* phosphate buffer (pH 2.5) and acetonitrile. The same mixtures of amino acids and NPAAs were separated using the packed columns under the same conditions as previously. Specifically, the separation

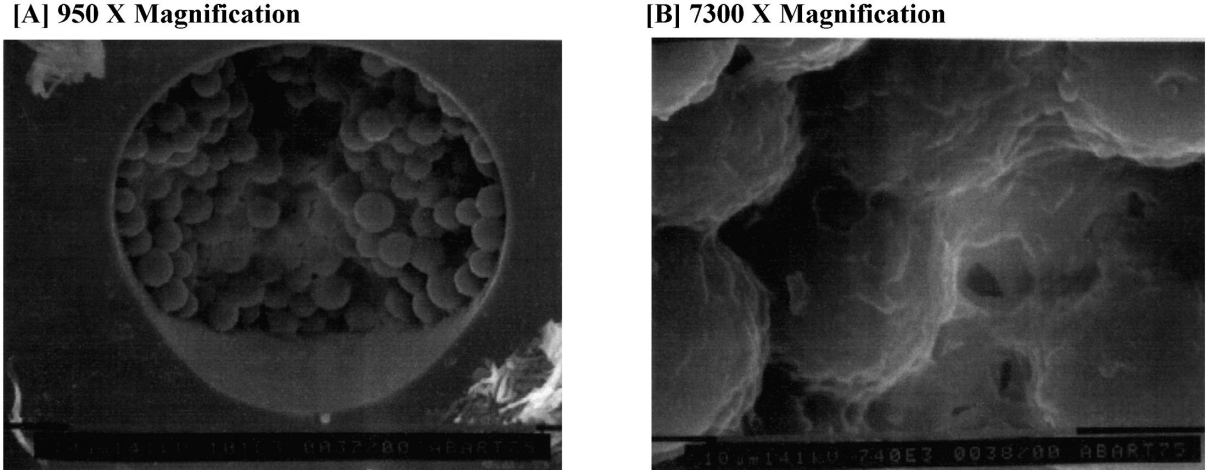

Fig. 3. Scanning electron micrographs of the inlet frit of the CEC capillary column.

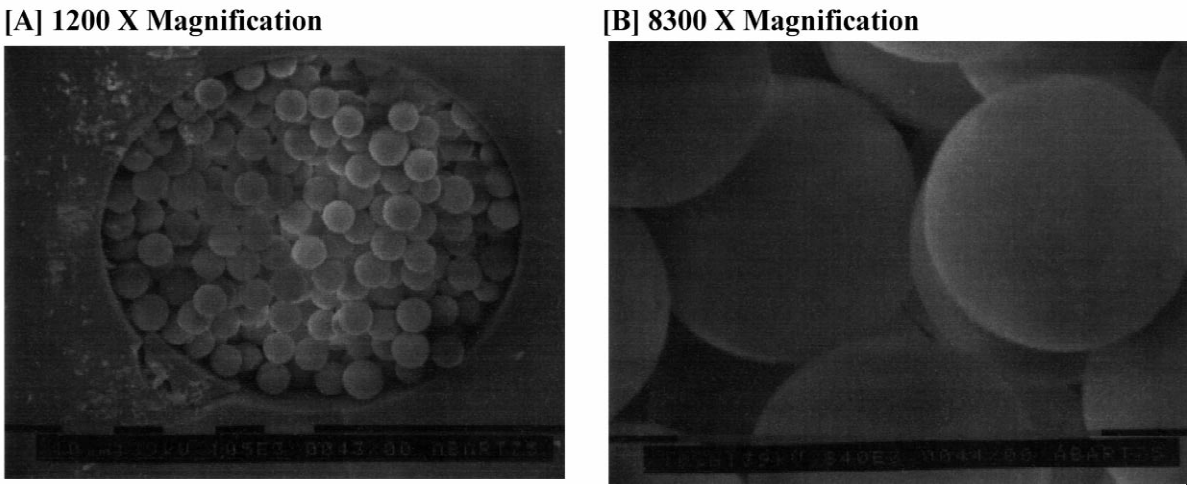

Fig. 4. Scanning electron micrographs of the packed section of the CEC capillary column.

solution is a mixture of 5 m*M* phosphate buffer (pH 2.5)–acetonitrile (30:70), the field strength is 0.50 kV/cm, and the temperature is 20°C. Table 1 lists the retention times, resolutions, elution orders, and plate heights of NBD-amino acids and NBD-NPAAs. Most of NBD-amino acids and NBD-NPAAs are eluted within 10 min, whereas NBD-glutamic acid (Glu) enantiomers are eluted in 40 min. In a packed column, the retention times of the amino acids are shortened as compared to the same separation in a particle-loaded monolithic column [28]. Under our experimental conditions, the electroosmotic flow is very small or negligible and electrophoretic velocity is the main driving force for analyte migration through the column. The separation solution and applied voltage of the packed column and the monolithic column are the same, so electrophoretic velocity of these analytes are similar between the packed column and the monolithic column. The different retention times between the two columns are derived from the different partitioning between the mobile and the stationary phases. Structural

Table 1
Elution time, resolution, and plate height for NBD-amino acid enantiomer separations

	Elution time for first eluted enantiomer (min)	Elution time for second eluted enantiomer (min)	Resolution	Elution order	Plate height for first eluted enantiomer (μm)
Alanine	8.28	8.35	4.01	D, L	8.7
Glutamine	7.23	7.97	2.63	D, L	14
Glutamic acid	39.72	42.20	1.21	D, L	20
Glycine	8.43				16
Isoleucine	6.92	8.10	4.41	D, L	12
Methionine	7.24	8.63	4.90	D, L	12
Phenylalanine	7.76	9.14	4.68	D, L	12
Proline	11.06	11.65	1.38	L, D	13
Serine	7.49	8.29	3.02	D, L	11
Threonine	6.24	6.91	2.77	D, L	13
Valine	7.40	8.54	5.03	D, L	11
2,3-Diaminopropionic acid	7.17	8.35	8.29	N.I	39
2-Aminobutyric acid	7.17	8.35	3.78	N.I	13
3-Aminobutyric acid	6.3	6.84	1.89	N.I	18

N.I.: Not identified.

differences between the packed and the monolithic columns contribute to the differences observed in the partitioning of the analytes.

Using the chiral column made with the photopolymerized sol–gel frits, all the NBD-amino acids and NBD-NPAAs were well resolved. The resolution factors are between 1.21 and 8.29. These values are about 1.5 times larger than those in the particle-loaded monolithic columns. The elution orders of the NBD-amino acids on the packed columns are the same as those on the monolithic columns [28], with the NBD-D-amino acids eluting faster than the corresponding NBD-L-amino acids, except for NBD-Pro. The elution orders of NBD-NPAAs were not confirmed, because the samples were made up of racemic mixtures rather than optically active ones. The plate heights for NBD-amino acids and NBD-NPAAs were less than 20 μm on the packed column, except for NBD-Glu and NBD-2,3-diaminopropionic acid. In the monolithic columns, the plate heights for NBD-amino acids and NBD-NPAAs are between 14 and 65 μm. These plate heights are about two times larger than those in the packed column. These NBD-Glu and NBD-2,3-diaminopropionic acid showed worse separations than other NBD-amino acids and NBD-NPAAs in both the packed and monolithic columns. In chromatographic separations, additional interactions that lead to reductions in the velocity of mass transfer increase the plate height of an analyte. Glu has two carboxyl groups that form ionic interactions with the unmodified amino groups of the aminopropyl silica gel. Two amino groups of 2,3-diaminopropionic acid are derivatized with NBD structures, making it different from the other amino acids and NPAAs. These two NBD structures might form some additional $\pi-\pi$ interactions with the packing particle. Therefore NBD-Glu and NBD-2,3-diaminopropionic acid showed worse separations than the others did in both packed and monolithic columns. The plate heights for all NBD derivatives, including NBD-Glu and NBD-2,3-diaminopropionic acid, are smaller for the packed column than the monolithic column.

Fig. 5A shows the electrochromatogram of a sample of NBD-DL-alanine (Ala) and NBD-DL-threonine (Thr) on the packed column, while Fig. 5B is the electrochromatogram of the same sample on the monolithic column. Similar elution times were

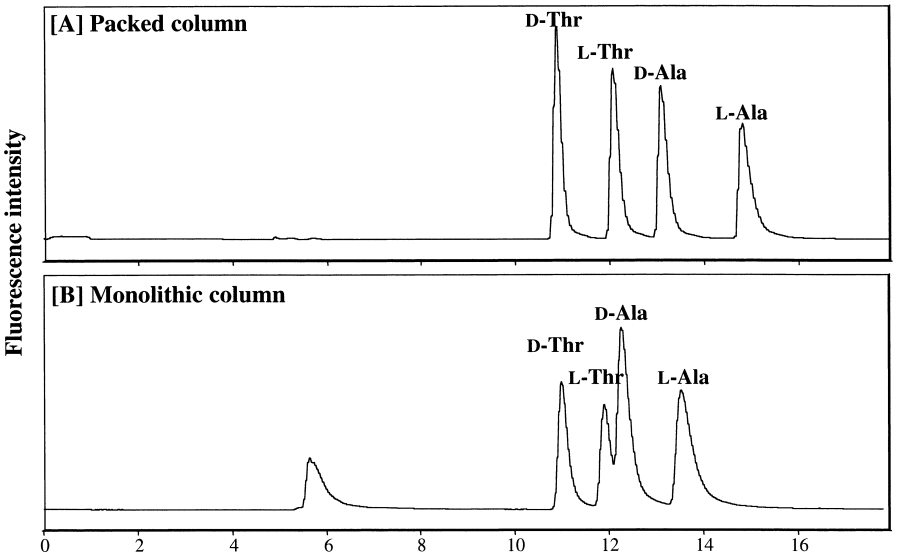

Fig. 5. Electrochromatograms of a mixture of DL-Thr and DL-Ala. Capillary: fused-silica capillary (30 cm×75 μm I.D.), packed segment; 15 cm long (packed with 5 μm chiral particles), mobile phase: mixture of 5 m*M* phosphate buffer (pH 2.5)–acetonitrile (30:70), field strength: (A) packed column at 0.50 kV/cm, and (B) monolithic column at 0.83 kV/cm.

achieved on the packed column as compared to the monolithic column by using an applied electric field of 0.5 kV/cm. A peak observed at about 6 min in Fig. 5B arises from the hydrolysis of the fluorogenic reagent [36]. The separation between the amino acids is vastly improved on the packed column. As seen in Fig. 5, baseline separations of the sample components were achieved with the packed column as compared to the monolithic column. The peak shape of each NBD-amino acid in the packed column is much sharper than in the monolithic column. These results show that the separation efficiency of the packed column is superior to that of the monolithic column.

The improvement in the separation efficiency and resolution of the sample of amino acids in the packed column as compared to the monolithic column may arise from better interaction of the amino acids with the chiral particles. In the particle-loaded monolithic columns, the particles may have been partially shielded as a result of encapsulation of the particles in the sol–gel matrix. In the absence of the sol–gel matrix, mass transfer is improved. Another reason for lower separation efficiency on the monolithic column might come from some heterogeneity in the sol–gel structure, such as small gaps or cracks. Such gaps or cracks occur as ethanol is evaporated from the reaction mixture during the thermal polymerization of the sol–gel used to embed chiral particles [28]. Photopolymerization allows us to avoid using heat, and consequently to avoid the formation of these gaps or cracks within the monolithic structure.

An additional advantage to using photopolymerized sol–gel to form frits is the ease and speed in preparation and the ease in controlling the length and the position of the frits as compared to the preparation of other photopolymerized or silicate frits [25,34,35]. A frit is made in 5 min upon exposure to UV light in our packed columns. The use of methacrylate-based reagents for frits [34,35] required 1–16 h of polymerization time. In case of silicate frits, only a few seconds are required for fabrication, but pretreatment of the capillary wall is required [25]. Consequently, preparation of packed capillaries using silicate frits requires an hour to fabricate the packed columns. Furthermore, it is more difficult to control the position and placement of frits that are prepared by heating.

Owing to the high porosity of the sol–gel frit, only 30 min at very low pressure (about 200 p.s.i. from a syringe on a hand-held vise) is required to pack a 15-cm section of chiral particles in the capillary. The backpressure is very low with the photopolymerized sol–gel frits as compared to silicate or photopolymerized methacrylate frits.

3.3. Performance of short-packed segment columns

In the packed column, the plate heights of the NBD-amino acid enantiomers are two-times smaller than the monolithic column. Consequently, NBD-amino acids are expected to be separated by a short packed column with a short separation time. Separation of NBD-amino acid enantiomers (NBD-Phe, -Val, -Gln, -Thr) are separated on a 5-cm packed segment column. The short packed column separates NBD-Phe enantiomers within only 5 min (Fig. 6). The separation factor for NBD-Phe enantiomers and the plate height for NBD-D-Phe are 2.22 and 8 μm, respectively. The plate height is improved on the short packed column, however the separation factor is decreased owing to the short packing segment.

4. Conclusions

An easy and fast method for the preparation of packed columns using photopolymerized sol–gel frits has been developed. Bubble formation is not observed during any of the chromatographic runs. NBD-amino acids and NBD-NPAAs were separated enantiomerically on a packed (15-cm segment) capillary using a mixture of 5 m*M* phosphate buffer, pH 2.5–acetonitrile (30:70) as a running buffer. Resolutions were between 1.21 and 8.29. These values are superior to those of our previous report (1.14–4.45) using a monolithic column with embedded chiral particles. The theoretical plate number for NBD-D-Ala is about 115 000/m, which is about 1.5 times better than in our previous report. Enantiomeric separation of NBD-Phe enantiomers within 5 min was possible on a 5-cm segment column. We find that photopolymerized sol–gel frits are advantageous in the construction of packed columns for CEC separations.

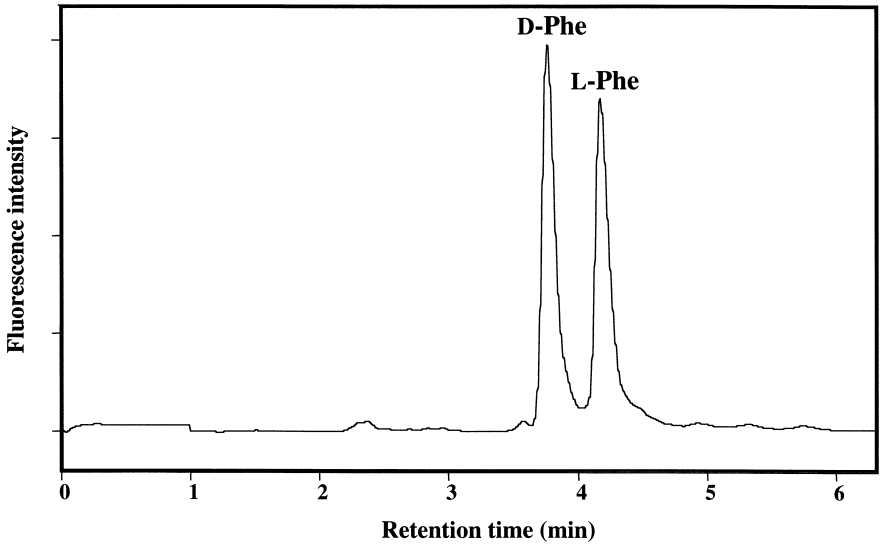

Fig. 6. Electrochromatogram of NBD-DL-Phe on a 5-cm packed segment column. Capillary: fused-silica capillary (26 cm×75 μm I.D.), packed segment; 5 cm length (packed with 5 μm chiral particles), mobile phase: mixture of 5 m*M* phosphate buffer (pH 2.5)–acetonitrile (30:70), field strength: 0.96 kV/cm.

Acknowledgements

We thank the Graduate School of Pharmaceutical Sciences, University of Tokyo and Sumika Chemical Analysis Service for supplying the chiral material. M.K. was supported by a postdoctoral fellowship from the Uehara Memorial Foundation (Tokyo, Japan). This work was supported by Beckman Coulter, Inc.

References

[1] V. Pretorius, B.J. Hopkins, J.D. Schieke, J. Chromatogr. 99 (1974) 23.
[2] J.H. Knox, I.H. Grant, Chromatographia 32 (1991) 317.
[3] M.T. Dulay, C. Yan, D.J. Rakestraw, R.N. Zare, J. Chromatogr. A 725 (1996) 361.
[4] N.W. Smith, M.B. Evans, Chromatographia 38 (1994) 649.
[5] F. Lelievre, C. Yan, R.N. Zare, P. Gariel, J. Chromatogr. A 723 (1996) 145.
[6] C. Yan, R. Dadoo, H. Zhao, R.N. Zare, D.J. Rakestraw, Anal. Chem. 67 (1995) 2026.
[7] K.D. Altria, N.W. Smith, C.H. Turnbull, J. Chromatogr. B 717 (1998) 341.
[8] C. Fujimoto, Trends Anal. Chem. 18 (1999) 291.
[9] M.G. Cikalo, K.D. Bartle, M.M. Robson, P. Myers, M.R. Euerby, Analyst 123 (1998) 87R.
[10] B. Chankvetadze, G. Blaschke, J. Chromatogr. A 906 (2001) 309.
[11] D. Wistuba, V. Schurig, Electrophoresis 21 (2000) 4136.
[12] M. Lämmerhofer, F. Svec, J.M.J. Frechet, W. Lindner, Trends Anal. Chem. 19 (2000) 676.
[13] T. Tsuda, K. Nomura, G. Nakagawa, J. Chromatogr. A 248 (1982) 241.
[14] W.D. Pfeffer, E.S. Yeung, J. Chromatogr. A 557 (1991) 125.
[15] J.J. Pesek, M.T. Matyska, J. Chromatogr. A 736 (1996) 255.
[16] Y. Guo, L.A. Colón, Anal. Chem. 67 (1995) 2511.
[17] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, J. Chromatogr. A 762 (1997) 135.
[18] E.C. Peters, M. Petro, F. Svec, J.M.J. Fréchet, Anal. Chem. 69 (1997) 3646.
[19] A. Palm, M. Novotny, Anal. Chem. 69 (1997) 4499.
[20] J. Liao, N. Chen, C. Ericson, S. Hjertén, Anal. Chem. 68 (1996) 3468.
[21] C. Fujimoto, Anal. Chem. 67 (1995) 2050.
[22] M.T. Dulay, R.P. Kulkarni, R.N. Zare, Anal. Chem. 70 (1998) 5103.
[23] G. Chirica, V.T. Remcho, Electrophoresis 20 (1999) 50.
[24] Q.L. Tang, B.M. Xin, M.L. Lee, J. Chromatogr. A 837 (1999) 35.
[25] M. Qi, X.-F. Li, C. Stathakis, N.J. Dovichi, J. Chromatogr. A 853 (1999) 131.
[26] B. Behnke, E. Grom, E. Bayer, J. Chromatogr. A 716 (1995) 207.
[27] R.J. Boughtflower, T. Underwood, C.J. Paterson, Chromatographia 40 (1995) 329.
[28] M. Kato, M.T. Dulay, B. Bennett, J.-R. Chen, R.N. Zare, Electrophoresis 21 (2000) 3145.

[29] B. Behnke, E. Grom, E. Bayer, J. Chromatogr. A 716 (1995) 207.
[30] R.A. Carney, M.M. Robson, K.D. Bartle, P. Mayers, J. High Resolut. Chromatogr. 22 (1999) 29.
[31] R. Asiaie, X. Huang, D. Farnan, Cs. Horváth, J. Chromatogr. A 806 (1998) 251.
[32] H. Rebscher, U. Pyell, Chromatographia 38 (1994) 737.
[33] H. Poppe, J. Chromatogr. A 778 (1997) 3.
[34] J.-R. Chen, M.T. Dulay, R.N. Zare, Anal. Chem. 71 (2000) 1224.
[35] C. Yu, F. Svec, J.M. Fréchet, Electrophoresis 21 (2000) 120.
[36] K. Imai, Y. Watanabe, Anal. Chim. Acta 130 (1981) 377.