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Enantiomeric separation of amino acids and nonprotein amino acids using a particle-loaded monolithic column

A solution is prepared of 5 μm silica particles modified with (*S*)-*N*-3,5-dinitrobenzoyl-1-naphthylglycine (particle **1**) or (*S*)-*N*-3,5-dinitrophenylaminocarbonyl-valine (particle **2**) suspended in liquid tetraethylorthosilicate, ethanol, and aqueous hydrochloric acid. This solution is injected under pressure into a 30 cm long, 75 μm inner diameter capillary column and heated for 1 h at 120°C after which the modified particles are embedded in a monolithic column of sol gel. The packed column measures approximately 15 cm from the inlet to the window used to view the laser-induced fluorescence. Thirteen different amino acids and three nonprotein amino acids are derivatized with the fluorogenic reagent 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) before injection onto the column for capillary electrochromatographic separation. The enantiomeric separation of the monolithic column packed with particle **1** results in a resolution ranging from 1.14 to 4.45, whereas that packed with particle **2** results in a resolution ranging from 0.79 to 1.17. On the basis of resolution and amount of chiral packing material the enantiomeric separation obtained by capillary electrochromatography is judged to be superior to that obtained previously with high performance liquid chromatography (HPLC).

Keywords: Amino acid / Nonprotein amino acid / Capillary electrochromatography / Sol gel / Enantiomeric separation
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1 Introduction

The analysis of biological samples is often complicated by the similarities in the chemical structures of the components of these samples. For example, there are twenty amino acids (AAs) that are used as the building blocks for proteins in mammalian tissue. Their structures differ only by the types of alkyl groups attached to a chiral carbon atom. Several nonprotein amino acids (NPAAs) were found to be misincorporated in the protein sequence. These include D-AAs with opposite chirality to L-AAs in mammalian tissue. These NPAAs and D-AAs are speculated to be related to autoimmune disease and to aging [1, 2]. To further understand the role of NPAAs and D-AAs in autoimmune diseases and aging, the determination of these NPAAs and D-AAs is required. The aim of our study was to analyze NPAAs and D-AAs in biosamples by means of capillary electrochromatography (CEC) using a chiral particle-loaded monolithic column with fluorescence detection for high sensitivity.

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Abbreviations: AA, amino acid; **NBD-F**, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; **NPAA**, nonprotein amino acids; **SEM**, scanning electron microscopy; **TEOS**, tetraethylortho-silicate

CEC [3, 4] appears to be a suitable method for the separation of chemically similar molecules. 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) [5–9]. There are numerous reports on the use of CEC for the separation of AAs, pharmaceutical drugs, and nucleic acids [10–12]. Chiral stationary phases (CSPs) have been used in CEC to separate the enantiomers of amines, alcohols, and AAs [7, 13–18]. CSPs can be classified into two types: (i) molecular imprinted polymers, and (ii) spherical particles modified with chiral selectors. Although capillary columns filled with molecular imprinted polymers are easy to prepare, they show low separation efficiency because of slow mass transfer of the polymer. Consequently, the range of separable compounds is small [18, 19]. Spherical particles, on the other hand, are "far superior" to imprinted polymers in that (i) high separation efficiency is accomplished using a small molecule selector, (ii) most of the chiral selectors are applicable regardless of their solubilities (chemical or physical attachment to silica gel is the only requirement), and (iii) a wide range of separable compounds exists.

Chiral selectors used to modify particles are proteins (α_1-acid glycoprotein, human serum albumin), cyclodextrins and their derivatives (β-cyclodextrin, hydroxypropyl-β-cyclodextrin, permethyl-β-cyclodextrin), cellulose deriva-

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tives, antibodies (vancomycins, teicoplanin), and small chiral molecules (quinine, naproxen) [7, 13–18]. With these chiral selectors, small molecules exhibit rapid mass transfer. Theoretical plate numbers of columns packed with these particles approach 200 000 per meter (plate height is 1.5 μm) [17]. We have prepared chiral particle-loaded monolithic columns using a sol-gel packing method [21]. The method involves the hydrolysis and condensation of metal alkoxide in water, alcohol, and catalyst solution. The sol-gel matrix embeds the particles and prevents them from exiting the column. The advantage of this particle-loaded monolithic column is that the amount of particles required for a CEC column is much less than that for an HPLC column. Only a few milligrams of the modified particles are needed for the preparation of a column for CEC. In HPLC, however, the amount of particles needed to pack a column is more than 100 times larger than that in CEC.

We analyzed enantiomers of AAs and NPAAs using these monolithic columns packed with 5 μm silica particles modified with chiral selectors, (*S*)-*N*-3,5-dinitrobenzoyl-1-naphthylglycine (particle **1**) or (*S*)-*N*-3,5-dinitrophenylaminocarbonyl-valine (particle **2**; Fig. 1). These chiral selectors were chosen because they showed rapid mass transfer in HPLC and separated AA enantiomers that are derivatized with a fluorogenic reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F; Fig. 1) [22–25]. Their resolutions in HPLC are between 0.70 and 2.00, and their theoretical plate numbers are about 20 000 per meter (plate height, 50 μm) [25].

2 Materials and methods

2.1 Apparatus

All the CEC experiments were performed using a commercial capillary electrophoresis system (Beckman model P/ACE System 5000; Fullerton, CA, USA) equipped with a laser-induced fluorescence (LIF) detector (P/ACE System Laser Module 488, λ_{ex} 488 nm). Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). Scanning electron microscopy (SEM) analyses were performed on a scanning electron microscope (Hitachi S-800, Tokyo, Japan).

2.2 Reagents

The 5 μm spherical chiral particles were donated by the Graduate School of Pharmaceutical Sciences, the University of Tokyo (Tokyo, Japan) and Sumika Chemical Analysis Service (Osaka, Japan). The 1 μm bare silica particle (nonporous) was purchased from Geltech (Miami, FL, USA). D- and L- AAs, D- and L- NPAAs, tetraethylorthosilicate (TEOS) and NBD-F were purchased from Sigma (St. Louis, MO, USA) or Aldrich Chemicals (Milwaukee, WI, USA) and were used as received. Millipore water was used in the preparation of all samples and buffers. HPLC-grade acetonitrile was purchased from Aldrich Chemicals and used without further purification.

2.3 Preparation of particle-loaded monolithic columns

A particle-loaded monolithic column was prepared using a previously described procedure [21] that has been

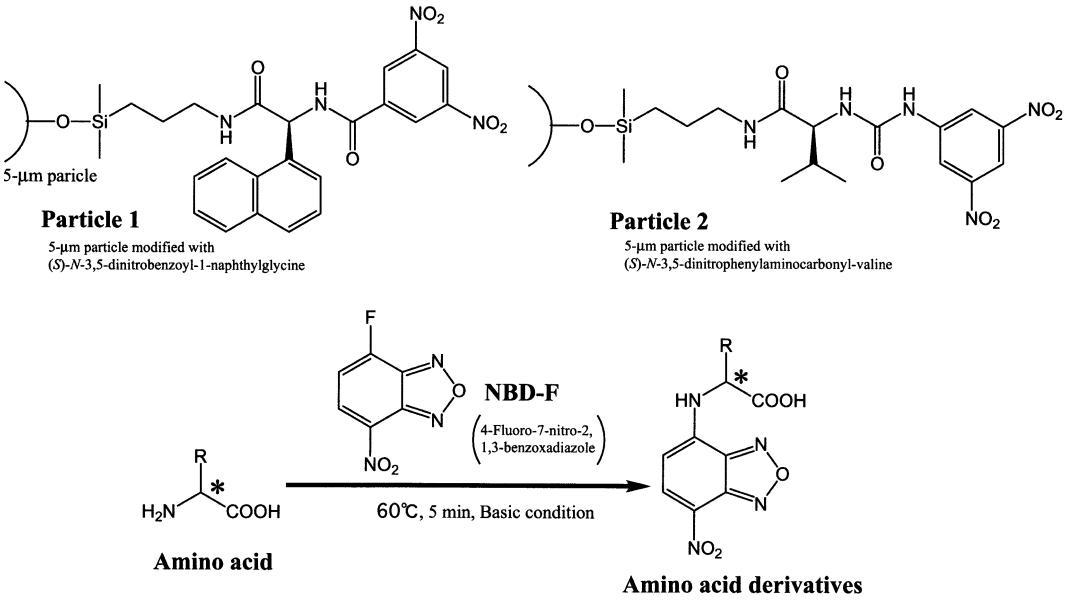

Figure 1. Chemical structure of packing particles and derivatization of an amino acid with a fluorogenic reagent.

modified to achieve a high density of packing particles in the capillary. Briefly, chiral particles were added at a concentration of 300 mg/mL to the sol-gel solution (mixture of 0.20 mL of TEOS, 0.73 mL of ethanol, and 0.10 mL of 0.12 M hydrochloric acid). Bare silica particles (1 μm) were added to the solution at a concentration of 4% w/w to improve and stabilize the electroosmotic flow (EOF). This solution was sonicated for several minutes and then introduced into a 75 μm inner diameter (ID) column of approximately 30 cm length by a syringe pressurized with a hand-held vise. During packing the particles stacked together at some point in the capillary. Packing was continued and the region beyond the initial stacking point was used to fabricate a window. A window was created on the column by using hot sulfuric acid (> 100°C) after the column was at 120°C for 1 h to facilitate ethanol evaporation. After heating, the column was preconditioned with running buffer that had been degassed by sonication (by pressurizing the column inlet to approximately 200 psi with a hand-held vise). Next, the column was further conditioned electrokinetically in the CE instrument by 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.

2.4 Derivatization of amino acids

A 10 μL volume of each 5 mM AA in 0.2 M borate buffer, pH 8.0, and 10 μL of 5 mM NBD-F in acetonitrile were mixed and heated at 60°C for 5 min. After addition of 20 μL of running buffer, the mixture was electrokinetically injected into the capillary (10 kV for 5 s).

2.5 CEC

A particle-loaded monolithic column with a packed segment of 15 cm from the inlet to the detection window was used. The derivatized sample was injected into the particle-loaded monolithic column electrokinetically at a temperature of 20°C. Applied voltages during the separations are mainly 0.83 kV/cm and 0.50 kV/cm for particles **1** and particle **2**, respectively. The analytes were observed by monitoring their fluorescence intensities.

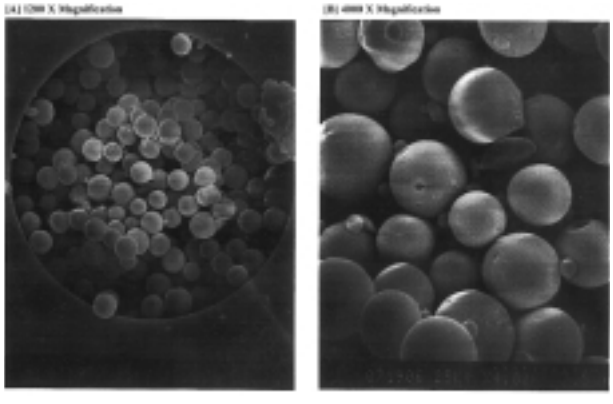

Figure 2. Scanning electron micrographs of CEC capillary column.

2.6 SEM analysis

Short segments (5 mm) of particle-loaded monolithic columns were sectioned and sputtered with gold for SEM analyses at an applied voltage of 25 kV.

3 Results and discussion

3.1 SEM study

A capillary packed with both particle **1** and bare silica particles was cut into 5 mm pieces for SEM analyses. As shown in the micrograph in Fig. 2A, the chiral particles and bare silica particles are fixed throughout the entire width of the capillary. The micrograph shown in Fig. 2B, an enlargement of Fig. 2A, shows a monolithic structure that is composed of both 5 μm chiral particle and 1 μm bare silica; however, there is no sol-gel matrix in which the particles are embedded. Here, the sol-gel acts to bond the particles together and to the capillary wall. In previous work from our laboratory [21] the chromatographic particles were embedded in a sol-gel matrix. The difference between these two monolithic structures was the density of the particles in the capillary, with the density being higher in the columns used for the experiments described here. When the particle density was increased, the volume of sol-gel solution in the capillary was lowered. The density of the packing is expected to affect the performance of the packed column, with a more densely packed capillary giving better separation efficiency than a less densely packed capillary.

3.2 CEC using particle 1

To avoid destroying the bond between the chiral selector and the silica particle, all separation runs were conducted using acidic and neutral running buffers (pH 2.5–7.5). Under acidic condition, the velocity of the EOF is very small or negligible compared to basic running conditions (pH 8–9). Hence, electrophoretic velocity is the main driving force for analyte migration through a CEC column. Because the NBD-AAs are negatively charged under our running conditions, they migrated toward the cathode. Therefore, the polarity of the electrodes was changed such that the anode was at the inlet and the cathode was at the outlet.

3.3 Effect of pH

We examined the effect of pH on the enantiomeric separation of NBD-alanine (Ala) using four different running buffers (pH 7.4 Tris buffer, and pH 6.5, 3.1, and 2.5 phosphate buffer). We used NBD-Ala as a typical NBD-AAs for experiments. At high pH, NBD-Ala derivatives were not eluted even after 1 h of separation. Variations in the v/v ratio of acetonitrile to running buffer did not affect the elution of NBD-Ala from the capillary at high pH conditions. At pH 2.5, Ala derivatives are rapidly eluted from the capillary because the low pH condition increased the electrophoretic velocity of NBD-Ala. Therefore, the best condition for separating NBD-Ala is with phosphate buffer at pH 2.5.

3.4 Effect of acetonitrile content

We studied the effect of acetonitrile volume in the running buffer (pH 2.5) on the separation of NBD-Ala (Table 1). The best separation of NBD-Ala was achieved with 70% acetonitrile in the running buffer. The retention times of the enantiomers were 13.9 min (D-form) and 15.2 min (L-form), and their resolution was 2.00. At higher volumes of acetonitrile in the running buffer, the separation efficiency is decreased. With 75% acetonitrile, the resolution of the enantiomers was 1.10, and the retention times increased to 16.7 (D-form) and 18.0 (L-form). When the volume of acetonitrile was greater than 80% in the running buffer, NBD-Ala enantiomers were not eluted within 1 h. At 60% acetonitrile in the running buffer, NBD-Ala enantiomers were rapidly eluted from the capillary (about 7 min), but did not separate enantiomerically. Because a high volume of acetonitrile is used in the running buffer, the electrophoretic velocities of the NBD-Ala enantiomers is reduced. As a result, the retention times of the enantiomers is increased. In addition, at high volumes of acetonitrile, NBD-Ala enantiomers are preferentially distributed in the mobile phase rather than on the particle surface. Therefore interactions between NBD-Ala enantiomers and CSPs are less favorable. Both retention times and partitioning of NBD-Ala enantiomers were related to their resolution. We find that 70% acetonitrile is a suitable condition for enantiomeric separation.

Table 1. Effect of running buffer for the elution profile for NBD-Ala

Phosphate buffer (mM)	Acetonitrile content (%)	Retention time for NBD-D-Ala (min)	Retention time for NBD-L-Ala (min)	Resolution for NBD-Ala
5.0	90	N.E.	N.E.	
5.0	80	N.E.	N.E.	
5.0	75	16.72	18.01	1.14
5.0	70	13.85	15.20	2.00
5.0	60	6.97	6.97	N.S.
7.5	70	12.09	12.99	1.44
10.0	70	8.12	9.07	1.19

N.E., not eluted within an hour
N.S., not separated

3.5 Effect of buffer concentration

Table 1 shows the retention times and resolution for NBD-Ala enantiomers under three different concentrations of phosphate buffer. High buffer concentrations, 7.5 mM and 10 mM, resulted in shortened retention times and poor resolution for NBD-Ala enantiomers. A high buffer concentration increases the current in the capillary as well as the electrophoretic velocities for NBD-Ala enantiomers, resulting in shortened retention times. This shortening of retention times reduces the interaction between NBD-Ala enantiomers and CSP, thereby decreasing their resolution. We find that the best separation is achieved with 5 mM phosphate buffer.

3.6 Separation with particle 1

Figure 3 shows the electrochromatogram of a sample of DL-threonine (Thr) and -glutamine (Gln). These four derivatized AAs were separated and eluted in this order: D-Thr, L-Thr, D-Gln, and L-Gln. Table 2 lists the retention time, resolution, elution order, and plate height of NBD-AAs, including NBD-NPAAs. Similar retention times were observed among these analytes, except for glutamic acid

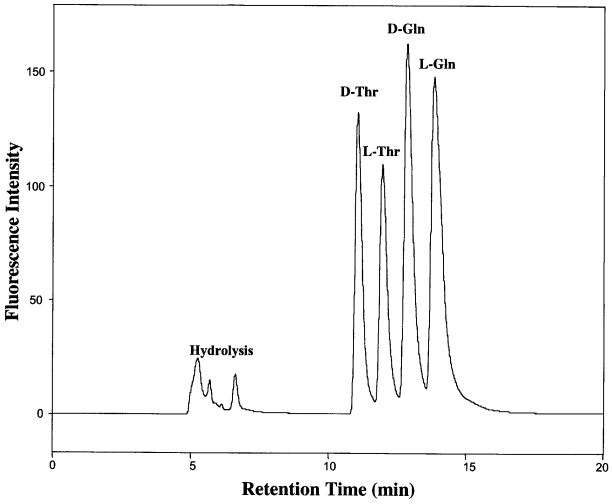

Figure 3. Electrochromatogram of DL-Gln and DL-Thr. Conditions: fused-silica capillary, 30 cm × 75 μm ID; packed segment, 15 cm length, packed with 5 μm chiral particle **1**; mobile phase, 5 mM phosphate buffer (pH 2.5): acetonitrile 30:70; field strength, 0.83 kV/cm.

Table 2. Results of separations with particle **1**

	Retention time for first eluted enantiomer	Retention time for second eluted enantiomer	Resolution	Elution order	Plate height for first eluted enantiomer (μm)	Elution buffer[a)]
Alanine	13.85	15.20	2.00	D, L	18	A
Glutamine	14.08	15.33	1.48	D, L	25	A
Glutamic acid	40.89	45.31	1.18	D, L	52	A
Glutamic acid	30.33	33.16	1.14	D, L	60	B
Glycine	16.26				28	A
Isoleucine	12.70	14.35	2.60	D, L	19	A
Methionine	13.27	15.26	2.52	D, L	22	A
Phenylalanine	14.01	16.13	2.88	D, L	20	A
Proline	18.15	19.20	1.20	L, D	28	A
Serine	13.20	14.60	1.85	D, L	25	A
Threonine	11.76	12.69	1.74	D, L	17	A
Valine	12.78	14.14	4.45	D, L	14	A
2,3-Diaminopropionic acid	16.27	20.40	2.33	N.I.	65	A
2-Aminobutyric acid	13.95	15.95	2.77	N.I.	22	A
3-Aminobutyric acid12.51	13.23	1.39	N.I.	13	A	

N.I., not identified

a) Elution buffers: A, 5 mM phosphate buffer (pH 2.5): acetonitrile 30:70, B, 7.5 mM phosphate buffer (pH 2.5): acetonitrile 30:70

(Glu) derivatives, which were eluted about 40 min under the same conditions. When the buffer concentration is greater than 5 mM, the retention times of Glu derivatives are drastically reduced, but still enantiomerically separable (Table 2).

Migration times of Glu derivatives are longer than those of other AA derivatives and these times are dependent on the buffer concentration. We suggest that the interaction between the carboxyl group of NBD-AAs and the unmodified amino group on the chiral particle be weakened because of phosphate ions binding to the amino group of the chiral particle. NBD-Glu has two carboxyl groups whereas the other AAs have only one carboxyl group. Because these carboxyl groups form an ionic interaction with the unmodified amino group of the particles, NBD-Glu was retained longer than the other NBD-AAs studied.

All NBD-AAs, except for NBD-Glu, showed baseline separation and their resolution are between 1.14 and 4.45. It was reported that two types of interactions, stereoselective and nonstereoselective interactions, were formed between chiral selectors and analyte. Stereoselective interactions lead to the separation of enantiomers, while nonstereoselective interactions decrease enantiomeric separation [26, 27]. In the case of Glu derivatives, an additional nonstereoselective interaction occurs between the unmodified amino group of the silica gel and the carboxyl groups of these derivatives. Therefore resolution for Glu derivatives is smaller than that for other NBD-AAs. The elution orders of these NBD-AAs were the same; that is, the D-form eluted faster than the corresponding L-form, except for proline derivatives. These results were the same as those obtained by HPLC [25]. The elution orders of NBD-NPAAs are not confirmed, because we had only racemic mixtures and not optically active ones. The plate height for each NBD-AA was about 20 μm, which is superior to HPLC studies [25] and CEC studies using protein, β-cyclodextrin derivatives, and molecular imprinted polymer as chiral selectors [7, 19, 20]. The plate height, however, was inferior to those obtained in CEC studies using 3 μm particles modified with naproxen [17]. It was reported that a smaller particle diameter improved the separation efficiency in chromatography [28–30]. Hence, we anticipate we can improve the separation efficiency of the particle-loaded monolithic column by reducing the particle size.

3.7 Separation with particle 2

We packed 5 μm silica particles modified with (*S*)-*N*-3,5-dinitrophenylaminocarbonyl-valine (particle **2**) into a capillary. The retention times for the AAs studied were decreased as compared to the columns packed with particle **1**. Under the same running conditions used with particle **1**, all NBD-AAs eluted from the column in about 10

min, and did not separate well enantiomerically. NBD-AAs formed weak interactions with particle **2** as compared to particle **1**. When 30% v/v of 5 mM phosphate, pH 3.0, and 70% acetonitrile (conditions that do not result in elution of NBD-AAs on particle **1**) were used, some NBD-AAs were separated (Table 3). Glu derivatives, however, did not elute even after 1 h, but did elute from the column at a higher field strength (0.67 V/cm). Of all the amino acids studied, NBD-Glu had the strongest interaction with particle **2**. Other NBD-AAs, including NBD-NPAAs eluted in about 15 min. Hydrophilic NBD-AAs, such as serine and threonine, separated well, while hydrophobic NBD-AAs, such as isoleucine, valine, and phenylalanine, did not separate at all. For all of the AAs studied, the D-form eluted faster than the corresponding L-form in the HPLC studies. The plate height (36–150 μm) for separable NBD-AA was worse than those by particle **1**, but better than in previous HPLC studies using particle **2** [25].

Although particle **2** separated all NBD-AAs and their resolutions were better than those by particle **1** in HPLC studies [25], particle **2** showed a poorer separation than particle **1** in CEC. Enantiomeric separation of NBD-AAs using particle **1** involved the formation of π-π interaction and hydrogen bonding [25]. A π-π interaction was formed between naphthalene of particle **1** and 2,1,3-benzoxadiazole of NBD-AAs. The strength of the π-π interaction appeared to be stronger in CEC than in HPLC, because a more hydrophilic solvent was used in CEC (30% 5 mM phosphate buffer, pH 2.5, and 70% acetonitrile) than in HPLC (5 mM citric acid in methanol). Because particle **2** lacks an electron-rich aromatic group, formation of a π-π interaction was not possible. Therefore enantiomeric separation by particle **2** was inferior to particle **1** in CEC.

3.8 Reliability

The reliability of each column was studied. None of the NBD-AA showed any difference in elution pattern over three months using a single column. Hence, the particle-loaded monlithic column for enantiomeric separation may be stable enough for the separation of biosamples.

4 Concluding remarks

A particle-loaded monolithic column for the separation of NBD-AAs and NBD-NPAAs was prepared using a sol-gel packing method. NBD-AAs and NBD-NPAAs were separated enantiomerically by a capillary packed with particle **1** using a mixture of 30% 5 mM phosphate buffer, pH 2.5, and 70% acetonitrile as a running buffer. Resolutions were between 1.14 and 4.45, these values being superior to HPLC results using column packing with particle **1**. Plate heights were between 14 and 65 μm, which was almost two times better than the HPLC results. We also prepared a particle-loaded monolithic column packed with particle **2**, which also separated NBD-AAs and NBD-NPAAs enantiomerically using a mixture of 30% 5 mM phosphate buffer, pH 3.0, and 70% acetonitrile. Resolutions and plate heights for separable NBD-AAs and NBD-NPAAs were between 0.34 and 1.17, and 36 and 150, respectively. These results were slightly better than HPLC results packed with particle **2**. Nonetheless, we find that particle **1** is superior to particle **2** for CEC. We therefore

Table 3. Results of separations with particle **2**

	Retention time for first eluted enantiomer	Retention time for second eluted enantiomer	Resolution	Elution order	Plate height for first eluted enantiomer (μm)	Applied voltage (kV/cm)
Alanine	13.96	15.37	1.15	D, L	54	0.50
Glutamine	17.06	18.23	0.79	D, L	60	0.50
Glutamic acid	>60	>60	–	–	–	0.50
Glutamic acid	40.29	42.51	0.34	D, L	150	0.67
Glycine	16.97				39	0.50
Isoleucine	14.24	–	–	–	94	0.50
Methionine	14.16	15.10	0.96	D, L	38	0.50
Phenylalanine	14.78	–	–	–	50	0.50
Proline	25.54	27.43	0.75	D, L	48	0.50
Serine	13.97	15.21	1.17	D, L	56	0.50
Threonine	14.00	14.87	0.91	D, L	36	0.50
Valine	14.07	–	–	–	79	0.50
2,3-Diaminopropionic acid	14.14	15.53	0.87	N.I.	83	0.50
2-Aminobutyric	14.42	15.28	0.75	N.I.	44	0.50
3-Aminobutyric acid	11.07	–	–	–	150	0.50

N.I., not identified

suggest that the particle-loaded monolithic columns for CEC prepared by sol-gel technology holds promise for enantiomeric separations.

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