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Focusing Review

Development and Pharmaceutical Applications of Functional Stationary Phases for Capillary Electrochromatography and Chiral Separation

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Abstract

We synthesized 3-(4-sulfo-1,8-naphthalimido)propyl-modified silica (SNAIP) for capillary electrochromatography (CEC). The unique structure of SNAIP contributed to the retention by three interactions including hydrophobic, electrostatic and π-π interactions and the acceleration of electroosmotic flow at an acidic condition. The CEC employing SNAIP was successively applied to the rapid separations of several drugs, peptides and polar compounds. Also, several examples for applying CEC to real sample analyses were demonstrated. Furthermore, with the idea to use adamantane as a shield to reduce the peak tailing of charged solutes in CEC, adamantyl (ADM)-functionalized polymer monolith by a single-step copolymerization with the monomer containing ADM structure and a cross-linker was presented. Three chiral stationary phases with different phenylalanine (Phe) peptide lengths, Phe_4, Phe_8, Phe_{12}, were prepared to study the effect of peptide length on enantioseparation in reversed-phase HPLC. The highest resolution was observed for the selector with intermediate peptide length (*i.e.*, Phe_8). The side chain of amino acids was also found to play a role for the separation performance of the chiral stationary phases (CSPs). The IR spectra suggested that the Phe peptides immobilized on the CSPs were assumed to be mainly in the α-helical state. Also, it was found that the conformation strongly contributed to the chiral recognition of the CSPs by thermodynamic study.

Keywords: Capillary electrochromatography; Chiral separation; Stationary phase

1. Introduction

Capillary electrochromatography (CEC), which combines the features of capillary zone electrophoresis (CZE) and high performance liquid chromatography (HPLC), is a powerful separation technique with high efficiency, high resolution, and low consumption of mobile phase and sample. The most commonly used stationary phase in CEC has been octadecyl silica (ODS), but in view of the need to maintain a high enough electroosmotic flow (EOF), it can only work in the mobile phase with relatively high pH. When mobile phases are used with low pH, the EOF decreases tremendously owing to the protonation of silanol groups on the surface of ODS and the inner wall of capillary. One way to overcome this problem is to use silica based mixed-mode stationary phase (C_{18}/ SCX) [1,2]. The benefit of using this type phase in CEC is the contribution of a high EOF due to the ionized sulfonic acid group even at low pH. El Rassi and co-workers pioneered the specially designed CEC column packed with mixed mode stationary phase which was composed of a hydrophilic, negatively charged sublayer (sulfonic acid) and a nonpolar top layer contained octadecyl ligands and presented its application for nucleic acids [3]. However, the synthesis procedure was multi-step and complex. Since the numbers of the stationary phases designed for CEC are still limited, such stationary phases are essential to show the potential of CEC and expand its applicability.

Enantiomers of a chirally active drug often have a dramatically different pharmacological or toxicological effect; therefore, the separation of enantiomers is very important. HPLC with a chiral stationary phase (CSP) is a popular method in the field of chiral separation because of

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its good reproducibility and preparative capability. The design and development of CSPs has attracted much attention for a number of years. To date, CSPs with several chiral selectors, such as macrocyclic antibiotics [4], polysaccharide derivatives [5], modified amino acid [6], protein [7] and cinchona alkaloids [8], have been reported and some of them are commercially available. However, these CSPs do not always give good enantioseparation of target analytes; therefore, successive efforts to develop a new type of CSP are required.

In this review, the developments of novel CEC stationary phase and CSPs with peptide chiral selectors are described.

2. Functional stationary phase for CEC

In the initially described CEC, the major focus was on the separation behavior of neutral pharmaceuticals and aromatic hydrocarbons that were well studied in reversed phase (RP)-HPLC. This was because these CEC employed HPLC stationary phases, *e.g.* ODS. In order to expand the applicability of CEC, the stationary phases which produce a stable EOF at wide pH range and rely other retention mechanism different from hydrophobic interaction. In practical, several workers employed ion exchangers as a stationary phase or mixed-mode phase and found them a viable alternative [9]. The π-π interaction is a type of electron donor-electron acceptor interaction, originating from π-electron systems in two unsaturated functional groups through intermolecular or intramolecular interaction [10]. The π-π interaction can successfully be utilized in the separation of closely related compounds like metabolites or degradation products [11]. Therefore, the retention based on π-π interaction should be included into the retention mechanism on CEC packing materials.

In this work, 3-(4-sulfo-1,8-naphthalimido) propyl-modified silyl silica gel (SNAIP, Fig. 1) was newly synthesized with the idea to use the fixed charge of sulfonic acid group as both the EOF generator and chromatographic retentive sites. It was easily prepared by a single reaction of aminopropyl silica (APS) with 4-sulfo-1,8-naphthalic anhydride at 150 °C for 5 h. The structure of SNAIP could contribute to the retention by three interactions including hydrophobic, electrostatic, and π-π interactions [12-14]. The influence of buffer pH on the EOF mobility (μ_{EOF}) was studied in the pH range of 2.5-7.5 using the mobile phase composed of 40% methanol in 5 mM phosphate (Fig. 2). By using SNAIP column, μ_{EOF} became 1.19 and 1.55 x 10^{-8} $m^2V^{-1}s^{-1}$ at pH 2.5 and 3.0, respectively. These corresponded to 1.29 and 1.43-fold increase of μ_{EOF} when compared with 3-(1,8-naphthalimido)propyl-modified silyl silica gel (NAIP) column. This increase was attributed to the introduction of sulfonic acid groups, which was imperative for a fast separation at low pH. As shown in Fig. 3, a rapid baseline separation of five barbiturates was achieved within 3 min by an applied voltage of 20 kV and a mobile phase of 40% methanol in 5 mM phosphate (pH 3.8). The prepared SNAIP column was applied for the separation of benzodiazepines at low pH and the separation mechanism of the basic compounds on SNAIP column was studied. The retention of each benzodiazepine decreased with increasing the buffer concentration. The decrease in the retention was presumably a result of weakening electrostatic binding of positively charged benzodiazepines to the negatively charged sulfonic acid groups. This explanation was supported by a linear relationship between the logarithmic values of *k* and phosphate concentrations, which is a typical behavior in ion-exchange chromatography [15]. On the other hand, in CEC with ODS (Hypersil C_{18}), Euerby *et al*. demonstrated that the elution order of studied barbiturates was completely consistent with their hydrophobicity and phenobarbital eluted before secobarbital even using a phenyl-bonded stationary phase with the retention by π-π interaction [16]. In CEC with NAIP, the elution order of four barbiturates (barbital, phenobarbital, secobarbital, and thiopental) was according to their hydrophobicity (Fig. 4). Although the retention of phenobarbital on NAIP increased compared with ODS due to π-π interaction between phenobarbital and NAIP.

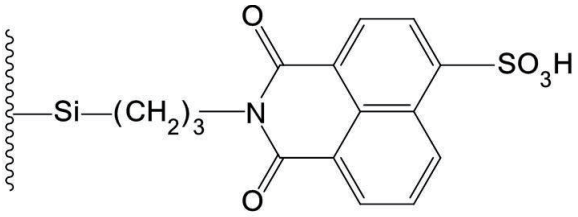

Fig. 1. Structure of SNAIP.

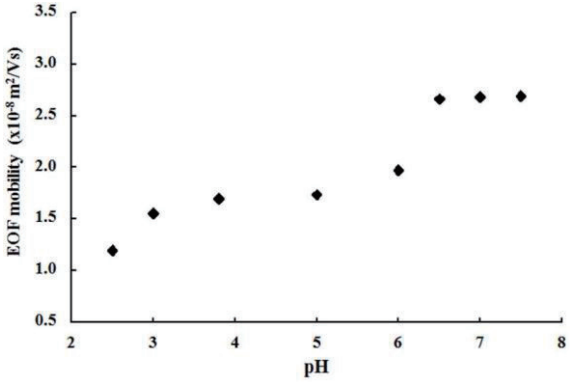

Fig. 2. Dependence of EOF mobility with SNAIP on the pH of mobilephase. Column, total length 370 mm (packed length 90 mm) x75 μm i.d.; mobile phase, 5 mM phosphate-methanol(60:40, v/v) at different pH values; applied voltage, 20 kV;temperature, 18 °C. Reproduced from ref. [12] with permission.

Contrastingly, in CEC with SNAIP, a different elution order was observed only for phenobarbital possessing an aromatic moiety that eluted last in spite of its lower log P value, while the order of the others was according to their log P values. In order to confirm the presence of π-π interaction, plots of log *k* against log P values for tested barbiturates were drawn. A linear relationship was obtained for tested barbiturates, except for phenobarbital. From these results, the difference in selectivity came from π-π interaction between SNAIP and the aromatic moiety of phenobarbital. Naphthalyl group modified with electron-acceptor group (*i.e.* sulfonic acid group) became π-acidic and thus the impact of the π-π interaction could be pronounced.

In RP-CEC, the separation of polar compounds (*e.g.* carbohydrates, nuleosides) requires the use of plain aqueous electrolytes solutions as mobile phases to obtain sufficient retention for polar compounds [17]. Unfortunately, like in HPLC, the highly polar mobile phase is unsuitable for a non-wetted non-polar stationary phase, which usually leads to high current and bubble formation within CEC column. In addition, when the buffer concentration modulating electrostatic interaction is too high, the bubbles can be generated within a capillary column by Joule heating, which hinders the possibility of gradient of buffer concentration. This bubble formation limits the use of wide variety of mobile phases, resulting in reducing the applicability of CEC. In this context, SNAIP is a relatively polar RP phase and therefore, using SNAIP column, an aqueous mobile phase would be available for CEC without bubble formation. In actual, no bubble was observed within SNAIP column even when the current reached 40 μA. This remarkable characteristic will expand the range of aqueous mobile phase composition and buffer concentration. At first, the effect of methanol content in the range from 5 to 40% (v/v) on the separation of nucleosides and nucleic acid bases was studied while keeping the buffer concentration at 10 mM and the pH of 5.0. An increase in EOF with a decrease in methanol content was obtained; the EOF mobility increased about 65% when the methanol content decreased from 40 to 5%. This tendency provides a significant advantage of suppressing the longer separation time with lowering methanol content that is generally used for better resolution in RP chromatographic separation. In fact, as shown in Fig. 5, the separation of early eluting peaks, namely uridine, uracil and thymidine, was improved by the decrease of methanol content; besides, the separation time for all analytes, except for adenine, was hardly prolonged. Furthermore, bubble formation was never experienced even under highly aqueous condition (~95% aqueous solution) of mobile phase. Next, the effect of buffer concentration on the CEC separations of peptides as well as protein digests

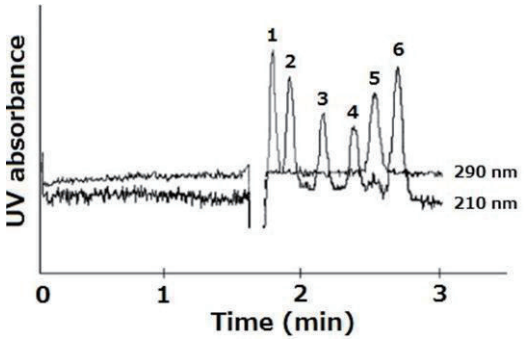

Fig. 3. Electrochromatogram of barbiturates with SNAIP. Mobilephase: 5 mM phosphate (pH 3.8)-methanol (60:40, v/v %). Other conditions as in Fig. 2. Peaks: 1, thiourea; 2, barbital; 3, amobarbital; 4, secobarbital; 5, thiopental; 6, phenobarbital. Reproduced from ref. [12] with permission.

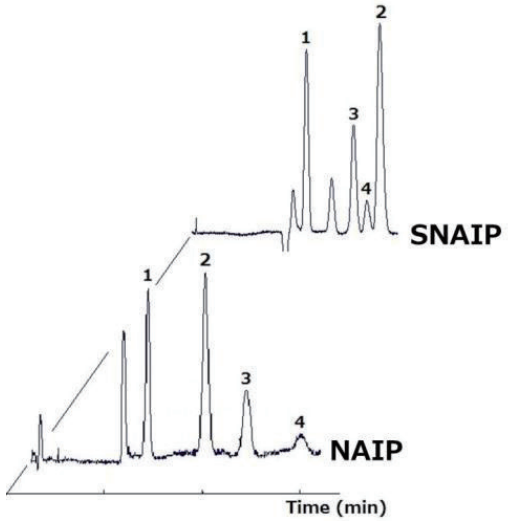

Fig. 4. Electrochromatographic separation of barbiturates with SNAIP and NAIP. Stationary phase, SNAIP; mobilephase, 5 mM phosphate(pH 3.8)-methanol (60:40, v/v %); applied voltage, 10 kV. Stationary phase, NAIP; mobile phase, 1 mM citrate (pH5.0)-methanol (60:40, v/v %); applied voltage, 20 kV. Other conditions as in Fig. 2. Peaks: 1, thiourea; 2, phenobarbital; 3, secobarbital; 4, thiopental. Reproduced from ref. [12] with permission.

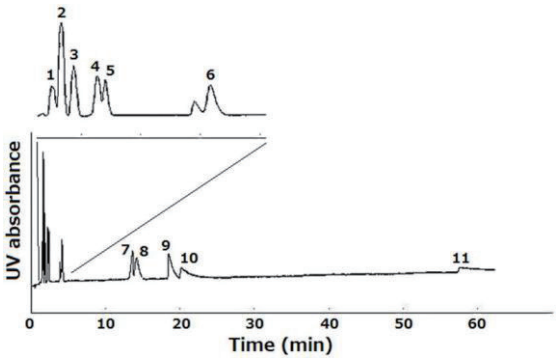

Fig. 5. Electrochromatographic separation of nucleosides and nucleic acid bases on SNAIP. Mobile phase, 10 mM phosphate buffer (pH 5.0)-methanol (90:10,v/v %); applied voltage, 15 kV. Other conditions as in Fig. 2. Peaks: 1, uridine; 2, uracil; 3, thymidine; 4, thymine; 5, inosine; 6, guanosine; 7, adenosine; 8, cytidine; 9, guanine; 10, cytosine; 11, adenine. Reproduced from ref. [14] with permission.

was investigated [18]. Fig. 6 illustrates the effect of buffer concentration on the separation of the tryptic digests of cytochrome *c* with the SNAIP column. Both mobile phases provided a sufficient separation and, as expected, increasing buffer concentration reduced the separation time from 130 min (20 mM, Fig. 6a) to 22 min (50 mM, Fig. 6b) and sharpened all peaks, especially later peaks. Using the mobile phase with 50 mM phosphate buffer, the resolution of tryptic digests on SNAIP column was superior or comparable to the pressurized gradient CEC in previous work [19]. This was because basic digests could be eluted by high buffer concentration decreasing electrostatic interaction between sulfonic acid groups on SNAIP and the digests. Based on these results obtained by the separation of various compounds, SNAIP can be a promising stationary phase for applying CEC to many separation scenes.

In order to gain a high reputation as practical technique, CEC must demonstrate an excellent performance, with respect to the separation speed, reproducibility and durability, in a number of real sample analyses. Barbiturates are widely used as sedatives, hypnotics and antiepileptics. Since barbiturates have a narrow therapeutic index, their improper use sometimes results in accidental death and also barbiturates are implicated in murder or suicide. For these reasons, the analysis for their presence in biological materials is frequently required. Here, CEC with NAIP was employed for the analysis of barbiturates extracted from human serum by liquid-liquid extraction, as shown in Fig. 7 [20]. The analysis of barbiturates in serum was successfully performed, with good repeatability of peak response (relative standard deviation with 11.1% for within-day and between-day) and short separation time in less than 4.5 min. Microdialysis is extensively used for continuous monitoring of substances in the extracellular space of tissues and body fluid. The advantage over other sampling methods is that microdialysates are protein-free, allowing direct analysis. The rapid separation obtained by CEC has the biggest advantage in such direct analysis; therefore, CEC with NAIP was applied for the analysis of caffeine and its metabolites (1-methylxanthine and 1,7-dimethylxanthine) in microdialysate [21]. A typical electrochromatogram of the microdialysates are shown in Fig. 8. The separation of caffeine and its two metabolites was achieved in less than 3.5 min by CEC with NAIP stationary phase, which was approximately 3-times less than that in HPLC with NAIP. The within-day repeatability as well as the between-day repeatability of the retention time and peak response were less than 5.9% (relative standard deviation). These examples support the good applicability of CEC for real sample analyses.

Organic polymer-based monolithic columns have been successfully used in CEC. Polymer-based monoliths are attractive because there are a wide variety of different monomers available and can be used within a broad pH-rage where they are chemically stable. They are prepared by a single-step copolymerization with monomer, cross-linker, pore-forming solvent and initiator. Moderately or strongly ionic monomers, *i.e.* 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), [2-(methacryloyloxy)ethyl]-trimethylammonium chloride, are extensively utilized in the preparation of polymer

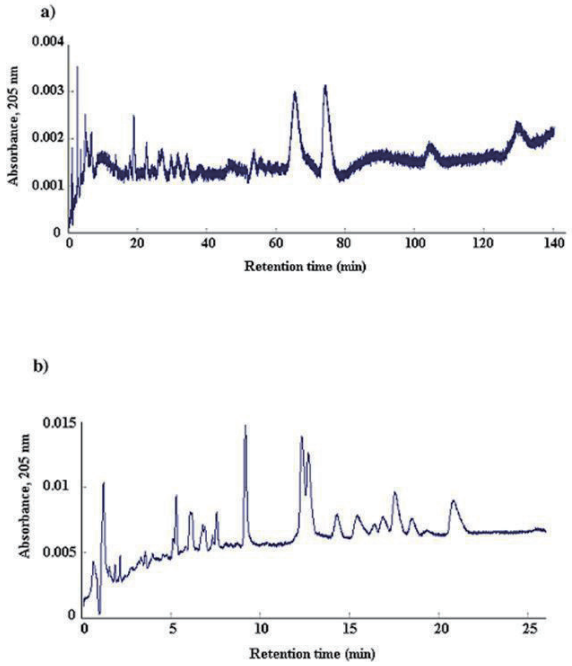

Fig. 6. Effect of buffer concentration on the separation of tryptic digests of cytochrome *c*. Mobile phase, phosphate buffer (pH 3.8)-methanol (60:40, v/v %) at different buffer concentrations: (a) 20 mM (27 μA); (b) 50 mM (95 μA); applied voltage, 20 kV. Other conditions as in Fig. 2. Reproduced from ref. [18] with permission.

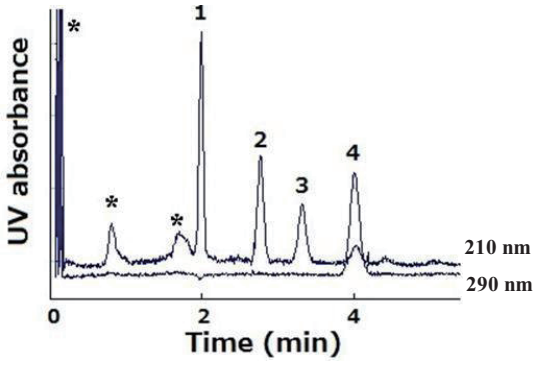

Fig. 7. Electrochromatogram of the serum sample spiked with barbiturates standards. Stationary phase, NAIP; column, 340 mm (packed length 90 mm) x 75 μm i.d.; mobile phase: 1.0 mM citrate buffer (pH5.0)-methanol (60:40, v/v %). Other conditions as in Fig. 2. Peaks: 1, barbital; 2, phenobarbital; 3, secobarbital; 4, thiopental; *, Peaks for intrinsic components in serum. Reproduced from ref. [20] with permission.

monoliths for CEC [22,23]. In the CEC separation of charged solutes, these monoliths work as a mixed-mode stationary phase that combines two retentive interaction (*i.e.* hydrophobic and cationic-exchange or anion-exchange). However, CEC of charged solutes on such stationary phases often suffers from severe peak tailing due to Coulombic attraction between solutes and monolith surface of opposite charges. In order to juggle production of stable EOF and prevention of peak tailing in CEC, new type of capillary monolithic columns should be developed. HPLC using the columns packed with silica-gel particles also suffers from peak tailing of basic solutes due to the interaction between solutes and silanols of packing material. In this context, some groups proposed the steric exclusion of the solute from the surface of silica-gel particles to reduce the interaction. Hemetsberger *et al.* observed that longer alkyl chains partially prevent solutes from reaching the underlying surface and play an important role in blocking the silanols [24]. Gilpin *et al.* and Guiochon *et al.* demonstrated improved peak symmetry for basic solutes by the bulky rigid structure of adamantane shielding the unreacted silanols from even small solutes [25,26]. With the idea to use adamantane as a shield to reduce the peak tailing of charged solutes in CEC, we newly synthesized adamantyl (ADM)-functionalized polymer monolith by a single-step copolymerization with the monomer containing ADM structure and a cross-linker [27]. Using a mobile phase of 5 mM acetate buffer (pH 4.0) and ACN mixture, the test solutes (aniline, *N*-methylaniline and *N*,*N*-dimethylaniline) were analyzed on ADM-functionalized and butyl methacrylate (BMA)-based monolithic columns made from the polymerization mixture consisting 23.9% 1-adamantyl-(α-trifluoromethyl) acrylate (MAF-ADE) or BMA, 15.9% ethylene dimethacrylate (EDMA), 0.4% AMPS, 6.0% water, 35.0% 1-propanol and 18.0% 1,4-butanediol. Table 1 shows retention factor and symmetry factor for the test solutes on both columns. The peak shapes were obviously better on ADM-functionalized monolithic column. The better peak shapes on ADM-functionalized monolithic column should be due to the reduction of the accessibility of basic solutes to sulfonic acid groups. This shielding effect of adamantane observed in polymer-based monolith conforms with that in the ADM-modified silica used for HPLC [25,26]. However, as shown in Table 1, the retention factor for basic solutes on ADM-functionalized monolithic column were lower than those on BMA-based one under the mobile phase conditions. This also supports the existence of the shielding effects which prevents the polymerization reaction and decreases the surface area of monolith.

In another approach to juggle production of stable EOF and prevention of peak tailing in CEC, methacrylic acid (MAA), a weakly ionic monomer, was employed in the preparation of BMA-based monolith for CEC in place of AMPS [28,29]. A weakly ionic monomer is an alternative; but the use of it is limited [30,31] because it is a weak EOF generator. Therefore, compared to AMPS, high content of MAA was employed in order to increase the ionizable functional groups (*i.e.* carboxylic acid groups) and then, strengthen EOF. Because polymer monolithic column containing high content of an ionic monomer is supposed to decrease its rigidity, less than 1.0% of ionic

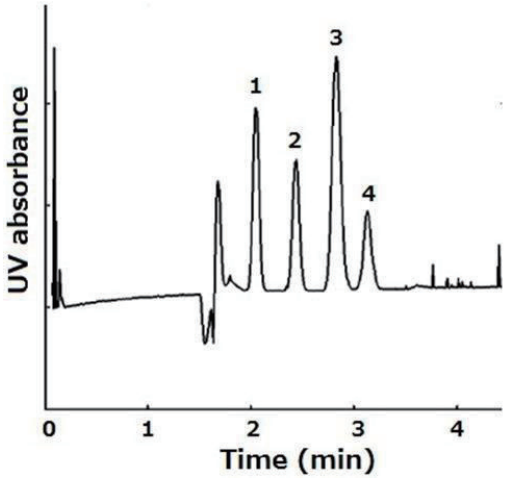

Fig. 8. Electrochromatogram of microdialysate sample-spiked with 25 μg/ml of caffeine and its two metabolites. Conditions: mobile phase, 4.0 mM citrate buffer-methanol (20:80, v/v %). Other conditions as in Fig. 7. Peaks: 1, 1-methylxanthine; 2, 1,7-dimethylxanthine; 3, pentoxifylline (E.S.); 4, caffeine. Reproduced from ref. [21] with permission.

Table 1. Peak shapes of basic solutes on ADM-functionalized and BMA-based monolithic columns[c].

	Aniline			*N*-Methylaniline			*N*,*N*-Dimethylaniline		
	RT (min)	*k*	*S*	RT (min)	*k*	*S*	RT (min)	*k*	*S*
ADM-functionalized monolith[a]	7.04	0.29	1.1	7.46	0.50	1.2	7.92	0.76	1.3
BMA-based monolith[a]	9.60	0.44	1.2	11.7	0.79	2.4	13.2	1.10	2.7
BMA-based monolith[b]	9.40	0.36	1.3	12.6	0.76	3.9	14.4	0.96	5.8

[a] 5 mM acetate buffer (pH 4.0)-acetonitrile (20:80, v/v %)

[b] 5 mM acetate buffer (pH 4.0)-acetonitrile (15:85, v/v %)

[c] Monolith (wt%): 23.6% MAF-ADE, 16.0% EDMA, 0.4% AMPS, 6.0% water, 35.1% 1-propanol, 18.9% 1,4-butanediol.

monomers were generally used [32,33]. Here, the effect of MAA content (0.05, 0.5 and 2.5%) on the CEC performance was studied with varying the composition of pore-forming solvents. It was noted that the higher MAA content provided the higher EOF mobility and the better column efficiency. In this study, the high MAA content is thought to swell the monolith than the low one does. Because of the high efficiency, the baseline separation of six test solutes was achieved in less than 100 seconds when using an applied voltage of 25 kV and the short-end injection method (9.0 cm capillary packed length).

3. Preparation and Characterization of poly(L-phenylalanine) chiral stationary phase

Since CSPs with a protein chiral selector have excellent enantioselectivity, a peptide chiral selector would seem to offer promise. Indeed, research on peptide chiral selectors has progressed. Li *et al.* developed polyproline-based CSPs and demonstrated their broad enantioselectivity in normal-phase mode [34]. Also, Chen used poly(*N*-benzyl-L-glutamine)-coated silica for the chiral separation of hydantoins [35]. We developed a novel CSP with phenylalanine (Phe) peptide as chiral selector [36-38]. The CSP was prepared on APS by solid-phase synthesis with *tert*-butoxycarbonyl (Boc) amino acids. N-terminus of all the CSPs were end-capped by benzoyl chloride (BZ). For a peptide chiral selector, the secondary structure introduces more conformational rigidity into the selector and can influence its enantioselectivity. The secondary structure of a peptide varies by its length [39], thus in this study CSPs with different peptide lengths were also individually prepared. At first, we studied peptide chiral selectors made from Boc-phenylglycine (Phg), Boc-valine (Val) and Boc-proline (Pro) in order to find out the kind of amino acid that is favorable for this class of CSPs. Warfarin enantiomers were used as a model chiral analyte in order to compare the newly synthesized CSPs, *i.e.* APS-$(Phg)_8$-BZ, APS-$(Val)_8$-BZ and APS-$(Pro)_8$-BZ, with APS-$(Phe)_8$-BZ under RP-mode. Enantioseparation was achieved on APS-$(Val)_8$-BZ (k_A, 19.2; k_B, 20.1; α, 1.05; *Rs*, 1.26) and APS-$(Phe)_8$-BZ (k_A, 27.3; k_B, 29.2; α, 1.07; *Rs*, 1.60) while no chiral resolution was observed on APS-$(Phg)_8$-BZ and APS-$(Pro)_8$-BZ. This result suggests that the side chain of amino acids plays a role for the separation performance of the CSPs. Phg and Phe differ only by the presence of the methylene group between α-carbon and phenyl group; this methylene group probably gives a high steric flexibility to the interactive site (*i.e.* phenyl group) with analytes. In this context, the side chain of Val is also sterically flexible. Pro does not have a flexible interaction site and thus, may not be suitable for our proposed CSPs. Besides warfarin enantiomers, the CSPs were used for the enantioseparation of trihexylphenidyl and benzoin which were partially separated on APS-$(Phe)_8$-BZ. However, no chiral separation was observed on the three CSPs. This can be because the side chain of Phe peptide works as a flexible and strong interactive site in the chiral recognition. Next, we investigated the effect of Phe peptide length (CSP**1**, Phe_4; CSP**2**, Phe_8; CSP**3**, Phe_{12}) on chiral separation in HPLC. Investigation of the chemical structure of the Phe peptide reveals that a combination of hydrophobic

Table 2. Chiral separation of *R*/*S*-wafarin on the CSP**1-3**.

		k_A	k_B	α	*Rs*
APS-(Phe)n-BZ	n=4 (CSP**1**)	20.1	20.8	1.04	1.03
	n=8 (CSP**2**)	27.3	29.2	1.07	1.60
	n=12 (CSP**3**)	31.0	32.7	1.05	1.31

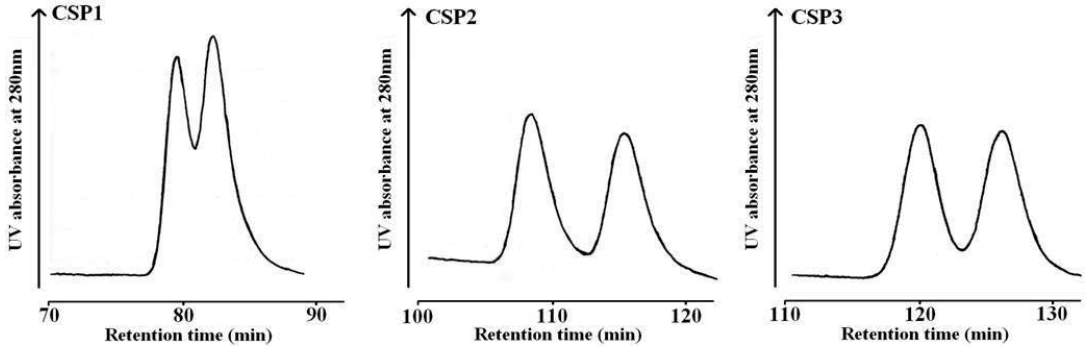

Fig. 9. Typical chromatographic separations of *R*/*S*-warfarin with CSP**1-3**. Mobile phase: 0.5M sodium perchlorate (pH 2.0)-acetonitrile (77:23, v/v %). Reproduced from ref. [36] with permission.

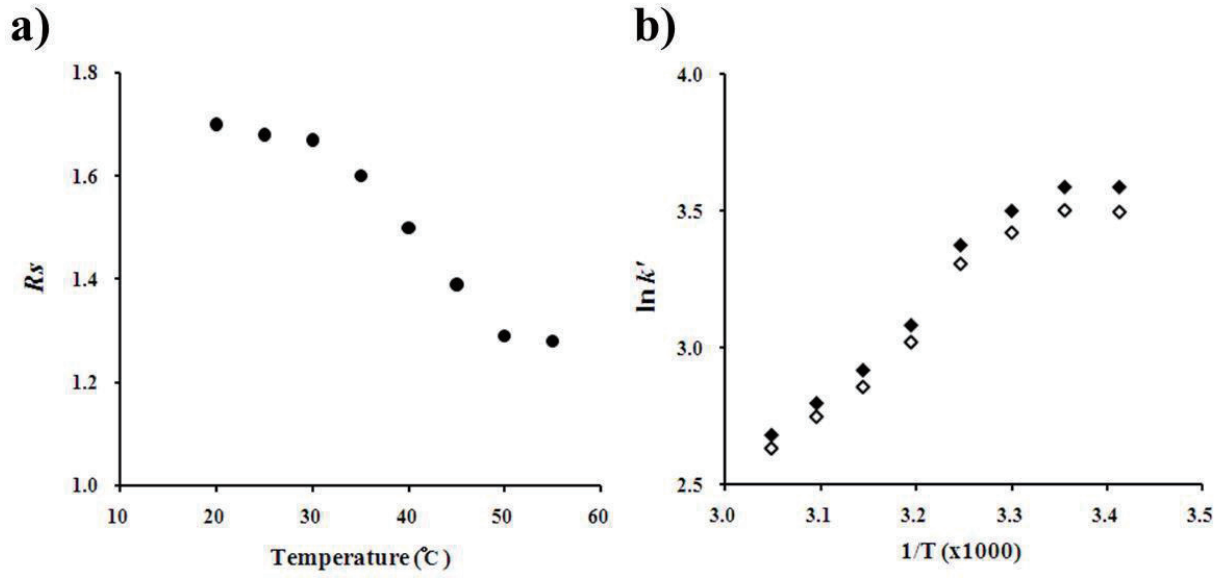

Fig. 10. a) Effect of column temperature on resolution factor and b) van't Hoff plot of warfarin enantiomers. Mobile phase: 0.5 M sodium perchlorate (pH 2.0)-acetonitrile (77:23, v/v %). Reproduced from ref. [38] with permission.

interaction, π-π interaction and hydrogen bonding would seem to contribute to the retention mechanism. Accordingly, a longer peptide chiral selector was expected to be more effective on the chiral separation because of increased hydrophobicity as the Phe peptide length increases and in general, the chiral recognition based on the secondary structure of peptide becomes more pronounced. The parameters (*k*, α, *Rs*) and the representative chromatograms in the separation of *R*/*S*-warfarin on CSP**1**-**3**, using a mobile phase consisting of 0.5M aqueous sodium perchlorate (pH 2.0) and acetonitrile (77/23, v/v), are shown in Table 2 and Fig. 9, respectively. Surprisingly, best resolution was achieved by CSP**2**, which had an intermediate peptide length. The same tendency was observed for the aminobutyl silica-based CSP having the same chiral selectors as CSP**1**-**3**. By making the Phe peptide selectors longer, hydrophobic interaction and π-π interaction are enhanced. The hydrogen bonding may be decreased, but its influence on the chiral recognition is relatively weak. Therefore, the lower enantioselectivity of CSP**3** than that of CSP**2** could be attributed to the secondary structure of Phe_{12}. The secondary structure of a peptide depends on its chain length [39] and variation in secondary structure could impact on enantioselectivity. An IR spectra is widely used to estimate the conformation of peptides and proteins from their amide I and II absorption bands [40,41]. IR measurement on CSP**1**-**3**, initially dispersed in the mobile phase for 12 h and then dried in vacuo at ambient temperature, was carried out. The resultant IR spectra provided an amide I absorption band at 1645-44 cm^{-1} and an amide II absorption band at 1541-35 cm^{-1}. Absorption bands at 1650-40 and 1550-20 are characteristic of α-helical structure [40,41] and thus the Phe peptides immobilized on CSP**1**-**3** were assumed to be mainly in the α-helical state. Therefore, the lower enantioselectivity of CSP**3** compared to CSP**2** could not be explained by differences in secondary structure. In aqueous solution, the major environmental factor that could affect the energetics of the helix-coil transition of peptides is temperature. Therefore, the contribution of column temperature to the chiral recognition of the poly(L-Phe) CSP was investigated using warfarin enantiomers as a model. Fig. 10a shows dependence of the resolution factor of warfarin on column temperature. Resolution factor decreased as temperature increased. Especially, it dramatically decreased when temperature increased from 30 to 50 °C. Furthermore, van't Hoff plot, which is the thermodynamic relationship between retention factor and temperature in Kelvin, was not linear (Fig. 10b). Non-linear van't Hoff plots may reflect a change in the relative contributions by enthalpy and entropy of transfer of the solute from the mobile phase to the stationary phase [42]. The changes in these relative contributions and possible changes in the retention mechanism are often thought to be related to conformation changes in the stationary phase (*e.g.* C18 stationary phases) [43]. Ma *et al.* reported that the α-helical conformations of polycationic peptide melt into extended ones as the temperature increased (from 1 to 60 °C) [44]. Similarly, Muñoz and Serrano reported a decrease of helical content as increasing temperature using polyalanine peptide [45]. From these observations, the chiral recognition of the poly(L-Phe) peptide selector was sensitive to the column temperature probably due to change of its secondary structure. We

further evaluated the enantioselectivity of the CSPs in normal phase (NP)-LC using four chiral analytes. The best resolution was obtained for benzoin enantiomers and as *n*-hexane content increased, the resolution factor increased due to the increase in NP mode retention. In RP mode, enantioseparation could not be achieved for mandelic and tropic acids, however partial separation was possible in NP mode, reciprocally warfarin which could be fully separated in RP mode [36] could not be separated in NP mode. The separable chiral analytes on this class of CSPs were changeable by the separation mode. In contrast to RP-LC, the current study showed that only the CSP with the longest peptide selector (Phe_{12}) provided an enantioselectivity in NP-LC. This was mainly due to its relatively high hydrophilicity; additionally, the peptide secondary structure, which was pronounced by the hydrogen bonds facilitated in organic mobile phase, might contribute to the chiral recognition. The optimum peptide length of our proposed CSPs for enantioseparation should be varied according to the separation mode.

4. Conclusion

The experimental data have confirmed that the separation mechanism in CEC with SNAIP stationary phase was a hybrid of electrophoretic migration and chromatographic retention involving hydrophobic, electrostatic as well as π-π interactions. Using low pH mobile phase, the baseline separation of five barbiturates in less than 3 min was observed due to the higher EOF. SNAIP is easily prepared on a single reaction and shows a different selectivity than conventional RP stationary phases. The application of the SNAIP stationary phase for the CEC of a wide range of analytes including pharmaceuticals, amino acids, peptides, protein digests, nucleosides and nucleic acid bases. Furthermore, it is demonstrated that ADM-functionalized monolithic stationary phase can provide good peak shape of basic solutes because of its shielding effect.

A new class of CSPs with a different number of Phe units immobilized on APS have been prepared and evaluated. The longest Phe peptide selector did not demonstrate the best enantioselectivity as expected, but the highest resolution was achieved by the CSP having intermediate peptide length. This indicated that enhanced enantioselectivity is not always obtained by a longer peptide chiral selector. Therefore, when a different amino acid is used to develop a peptide chiral selector, optimization of peptide length can enhance enantioselectivity. The side chain of amino acids was also found to play a role for the separation performance of the CSPs. The IR spectra suggested that the Phe peptides immobilized on the CSPs were assumed to be mainly in the α-helical state. Also, it was found that the conformation strongly contributed to the chiral recognition of the CSPs by thermodynamic study.

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