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Open Tubular Molecular Imprinted Polymer Fabricated in Silica Capillary for the Chiral Recognition of Neutral Enantiomers in Capillary Electrochromatography

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In this study, we have expanded the applicability of the pre-established generalized preparation protocol to MIPs with a neutral template. The (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone MIP layer was formed inside a pretreated and silanized fused silica capillary, and its chiral separation performance was examined. Optimization of chiral separation was also carried out. This is the very first report of somewhat successful application of the generalized preparation protocol to a MIP with a genuine neutral template.

Key Words : Neutral template, Molecule imprinted polymer, Chiral separation, Open tubular capillary, Capillary electrochromatography

Introduction

Molecular imprinting is a procedure of forming an artificial receptor where a template molecule is introduced in a cross-linked polymer matrix. After the template is removed by exhaustive washing, the resultant polymer network possesses a permanent groove of the original template. The molecule imprinted polymer (MIP) shows favored affinity to the template molecule compared to other molecules, and this property is responsible for diverse application of MIP techniques. MIP techniques have been increasingly employed in a variety of applications such as separation, purification, sample pretreatment, sensors, catalysts, and drug delivery, *etc*. MIP materials have been prepared in various formats such as spherical or irregular micro-particles, nano-particles, monoliths fabricated in a stainless steel or capillary column, open tubular layers in capillaries, membranes, and composites, *etc*.

A recent comprehensive review[1] is recommended to interested readers for details of theoretical and experimental features of MIPs of various formats as well as their widespread application in a variety of fields. There have been some specific reviews on application of enantiomeric recognition of MIPs, too.[2,3] Reviews with emphasis on characterization, evaluation and optimization of MIPs have been offered by several groups.[4-7] In addition, recent representative review articles are recommended to interested readers for specific use of MIPs in SPE,[8] drug delivery,[9] artificial enzymes or receptors for antibodies,[10] and sensors.[11,12]

MIP techniques have also been extensively used in chromatography. The recognition properties of MIPs have been explored *via* high performance liquid chromatography (HPLC) and capillary electrochromatography (CEC), the majority of application being with CEC recently as revealed in recent reviews.[13-20] CEC makes use of both superior selectivity of HPLC and high separation efficiency of capillary electrophoresis (CE). Among the various formats used in CEC, open-tubular capillary electrochromatography (OT- CEC)-based MIP columns are promising tools for the research of recognition abilities of MIPs owing to the merits such as simple column preparation, no bubble formation, and stable EOF application, *etc*. Since the first successful attempt to prepare an open-tubular MIP-CEC column by Brüggermann *et al*.,[21] there have been continual studies especially in enantio-separation.[22-34]

Enatio-separation (chiral separation) by the MIP shows a predetermined elution order for a pair of enantiomers if one of them is imprinted in the matrix; the imprinted enantiomer elutes later. The major disadvantage of MIP chiral separation is the fact that a MIP column is in general useful for chiral separation of only the pair of template enantiomers and that different MIP preparation protocols are required for a new column to resolve another pair of enantiomers. The whole procedure to find a new optimized preparation protocol is time consuming, thus a generalized preparation protocol for a variety of templates is desperately required. Such a generalized preparation protocol for OT-CEC MIP capillary columns was recently developed in a series of studies by our group.[25-34] The templates of such studies were bases, acids, and their derivatives.

In this study, we tried to expand the applicability of the generalized preparation protocol to MIPs with a neutral template. We have had some difficulties to discover commercial neutral templates of good chiral recognition susceptibility without free amino or carboxylic groups, thus only one template has been selected in this preliminary study. The (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone MIP layer was formed inside a pretreated and silanized fused silica capillary, and its chiral separation performance was examined. Optimization of chiral separation was also carried out. This is the very first report of somewhat successful application of the generalized preparation protocol to a MIP with a genuine

neutral template.

Experimental

Chemicals and Materials. Fused-silica capillaries (50 μm ID, 365 μm OD) were purchased from Grace (Deerfield, IL, USA). Methacrylic acid (with 100 ppm hydroquinone), ethylene glycol dimethacrylate (with 100 ppm hydroquinone monomethylether), γ-methacryloxypropyl trimethoxysilane (γ-MAPS), sodium salt of 4-styrenesulfonic acid (4-SSA), glacial acetic acid, sulfuric acid, sodium phosphate, (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone were obtained from Sigma-Aldrich (St. Louis,MO,USA) and (4*R*,5*S*)-4-methyl-5-phenyl-2-oxazolidinone was obtained from Fluka (Zwijndrecht, Netherlands). Azobisisobutyronitrile was purchased from Junsei Chemical (Tokyo, Japan). HPLC grade acetonitrile (ACN) and water were obtained from SK Chemicals (Ulsan, Korea). All of the reagents except for 4-styrenesulfonic acid (sodium salt hydrate) were used as received.

A modified procedure of acidification of 4-SSA salt was used in comparison with the previous study.[25] Thus the sodium salt hydrate of 4-styrenesulfonic acid (100 mg) was added to a mixture of 1 mL water and 13 μL sulfuric acid, and stirred well till complete dissolution. The solution was evaporated to dryness and the residue was stirred with 30 mL acetonitrile at room temperature overnight. The resultant mixture was settled down and the supernatant layer was decanted and collected. The residue was extracted with 30 mL acetonitrile once more and the supernatant was added to the previous aliquot. The collected supernatant was vaporized in a Rota-vapor at room temperature to give the solid product of 4-styrenesulfonic acid.

Aqueous phosphate buffers were prepared in 50 mM concentration by adjusting the mixing ratio of NaH_2PO_4 and Na_2HPO_4 to get the required pH according to the Handerson-Hasselbalch equation: $pH = 7.21 + \log (n_{Na_2HPO_4}/n_{NaH_2PO_4})$. The actual pH was measured and a small amount of 100 mM phosphoric acid or 100 mM NaOH was added to correct minor pH discrepancies.

CEC mobile phases were prepared by mixing the required volume of aqueous 50 mM buffer with the required volume of acetonitrile. For example, 2 mL 50 mM phosphate aqueous buffer at pH 7.0, and 8 mL acetonitrile were mixed to obtain 80/20 (v/v) acetonitrile/ phosphate buffer at pH 7.0.

Preparation of OT-MIP Capillary Column. The inner surface of fused silica capillary was activated by rinsing with 1 M NaOH for 6 h followed by washing with water, 0.1 M HCl, and water in sequence, and by drying with a flow of nitrogen. The capillary was then flushed with a solution of 4 μL γ-MAPS in 1 mL of 6 mM acetic acid for 6 h, and washed thoroughly with methanol to remove excess reagent and dried under N_2. Next, the MIP reaction mixture was filled into the capillary. The formulation of the MIP polymerization mixture was as follows: 0.02 mmol template (3.5 mg), 8.2 μL MAA, 59 μL EDMA, 2 mg 4-SAA, and 3.5 mg azobisisobutyronitrile (AIBN) in 1 mL of a 9/1 mixture (v/v) acetonitrile/2-propanol. 4-SSA is very hygroscopic demanding a special care. Once it is completely dehydrated in a dessicator, its weight is instantly measured and it is dissolved in a 9/1 mixture of acetonitrile/2-propanol at a concentration of 2 mg/mL. A tiny amount of residual inorganic salt can precipitate upon standing. In this case the supernatant is transferred to another bottle and stored with a tightened cap in a refrigerator at 4 °C. This solution is used for the preparation of the MIP reaction mixture.

The MIP reaction mixture was sonicated for 10 min, purged with nitrogen for 10 min, immediately filtered through a 0.25 mm syringe membrane filter, and filled in the silica capillary. Both ends of the capillary were plugged with rubber septa, and the capillary was immersed in a water bath for 4 h at 50 °C. Right after completion of reaction, the capillary was thoroughly washed with 90/10 methanol/acetic acid, and acetonitrile.

Instrumentation. Chromatographic data were obtained with an Agilent (Waldbronn, Germany) HP3D CE system with a diode array detector and the Chemstation data processing software. The stock buffer solution (50 mM sodium phosphate) was kept in a refrigerator, and the actual eluent was prepared by adding a determined amount of ACN. All analytical samples were made to a concentration of 1.0 mg/mL by dissolving in the mobile phase and stored in a refrigerator at 4 °C, and were further diluted (1/50 dilution) with the mobile phase for analyses. All the samples and eluents were filtered through a 0.2 μm cellulose membrane before analysis. Samples were injected hydrodynamically for 5 s under a pressure of 6 mbar. The detection wavelength was set to 214 nm. All the separations were carried out at a constant CE voltage of 30 kV and a temperature of 25 °C throughout. The effective length of all the MIP columns was 28 cm (total length; 36.4 cm).

Results and Discussion

Morphology of the (4*S*,5*R*)-4-Methyl-5-phenyl-2-oxazolidinone MIP. The SEM photograph of the (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone MIP layer produced on

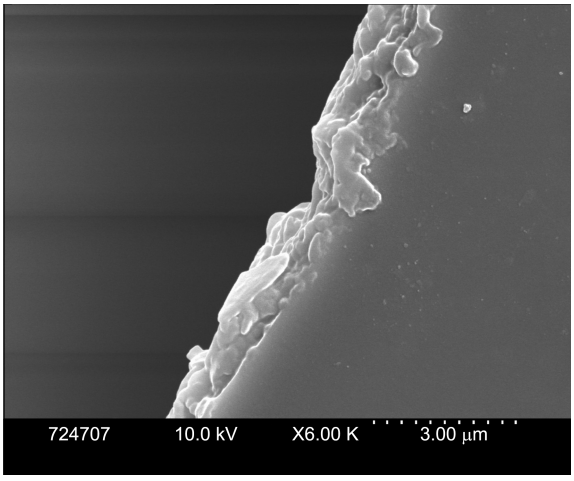

Figure 1. The SEM photo of the (4*S*,5*R*)-4-Methyl-5-phenyl-2-oxazolidinone MIP layer formed on the capillary inner wall.

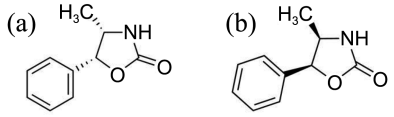

Figure 2. Molecular structures of (a) (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone, and (b) (4*R*,5*S*)-4-methyl-5-phenyl-2-oxazolidinone.

the inner wall of capillary is shown in Figure 1. The molecular structures of (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone and (4*R*,5*S*)-4-methyl-5-phenyl-2-oxazolidinone are drawn in Figure 2. The chiral separation performance of the MIP of this study is moderately good, however it is quite inferior to those of the MIPs with templates of high chiral recognition susceptibility such as profen drugs,[25,27,29] which will be discussed in more detail later.

Optimization of Enantiomeric Separation with Respect to pH. Optimization of eluent pH was carried out over the range of pH 6-8 making use of 50 mM phosphate buffer since pH 7.0 was expected to give the optimum results for a neutral template.[31] The procedure of pH optimization is illustrated in Figure 3. The optimum ACN composition was roughly estimated to be 80% in a preliminary experiment, thus 80% ACN was used throughout pH optimization.

In the pH range considered in this study, the acidic moieties (MAA and 4-SSA) of MIP are regarded to be almost dissociated (negatively charged), and the electro-osmotic flow (EOF) is expected to be virtually invariant with respect to pH. The pK_a of MAA is 4.65. The pK_a value of the analyte could not be found in the literature. Instead the pK_a value of an analogous compound ((*S*)-4-benzyl-2-oxazolidinone) was reported to be 12.78 in an internet web site (http://www.chemicalbook.com/Product MSDS DetailCB5721531_EN.htm). The pK_a value of (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone seems to be similar to this value. Thus, the analyte is also assumed to be virtually uncharged over the pH range of 6-8. If so, virtually no change either in retention times or in chiral separation performances should be observed with respect to pH. The fact is that some changes in retention times and variations of chiral separation were observed as shown in Figure 3. As the pH was increased from 6 to 8, the analyte retention times were increased. This cannot be explained by the change of dissociation fraction of the acidic moieties if any because the pH change from 6 to 8 would have caused reduction of retention times owing to increased dissociation of MIP acidic moieties and consequent increase of EOF. Some different explanation is necessary. It seems that the variations of relative amounts of ionic species with respect to pH should be considered. The total concentration of aqueous phosphate buffer was maintained 50 mM by varying the ratio between NaH_2PO_4 and Na_2HPO_4 to obtain the desired pH. Thus for the change of pH from 6 to 8, the concentration of HPO_4^{2-} and Na^+ will increase and the concentration of $H_2PO_4^-$ will decrease. The concentration of Na^+ in the eluent will be increased for the pH change from 6 to 8 to cause increased adsorption of Na^+ onto the MIP resulting in enhanced partial neutralization of the negative surface charge and consequent reduced EOF.

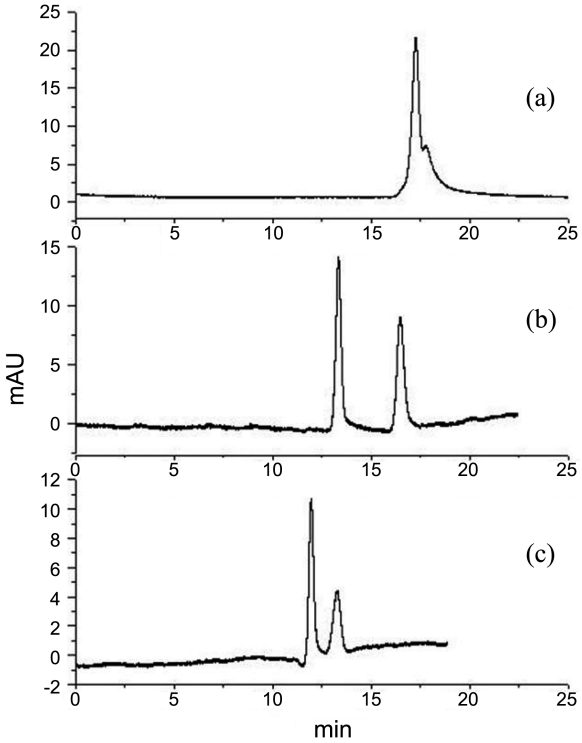

Figure 3. Influence of eluent pH on the separation of 4-methyl-5-phenyl-2-oxazolidinone enantiomers. (a) pH 8, (b) 7, (c) 6. First peak; (4*R*,5*S*)-4-methyl-5-phenyl-2-oxazolidinone, Second peak; (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone. <Experimental conditions> mobile phase, 80:20 (v/v) ACN/50 mM sodium phosphate; applied voltage, 30 kV; temperature, 25 °C; injection, 4 mbar 4s; detection wavelength, 214 nm.

The better chiral recognition at pH 7 than at either pH 6 or 8 shown in Figure 3 is somewhat difficult to explain. The pK_a value of the analyte is estimated to be close to 12.78 as mentioned before, and any information on its pK_b has never been reported in the literature. The analyte seems to be an extremely weak acid and base simultaneously (no ionization at pH 7.0). Assuming that the isoelectric point of analyte is 7.0 (neutral molecule), the analyte will have slight positive charge at pH 6.0, and slight negative charge at pH 8.0. We only guess that the analyte molecular structural alternation (conformation or resonance) upon bearing charges may be taken place to perturb the analyte-MIP recognition seriously, and this perturbation may be more severe when the analyte bears partial negative charge (pH 8.0). Anyway, it is common for all the MIPs that too high or too low a pH is harmful for efficient chiral recognition, and there should be an optimized pH midway.[25-34] The optimum pH of this study was found 7.0 as expected.

Optimization of Enantiomeric Separation with Respect to ACN Composition. The pH was maintained at pH 7 throughout optimization of ACN composition. As the acetonitrile content was increased in the eluent (70→80→92%), the analyte retention times were decreased as expected on the basis of enhanced EOF (Fig. 4). It is ambiguous to determine the optimum ACN composition based on Figure 4 since the optimum value for the best separation resolution

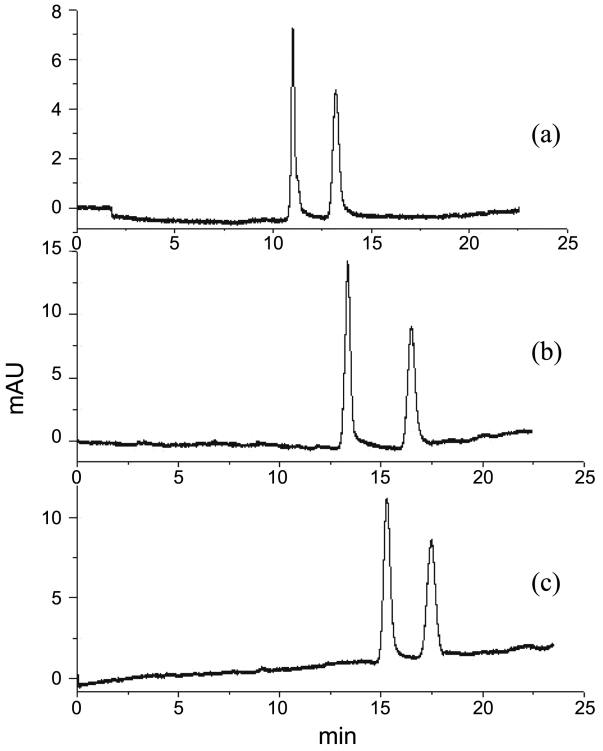

Figure 4. Influence of ACN composition on chiral separation of 4-methyl-5-phenyl-2-oxazolidinone enantiomers in various eluents with different ACN composition. (a) 92, (b) 80, (c) 70%. First peak; (4*R*,5*S*)-4-methyl-5-phenyl-2-oxazolidinone, Second peak; (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone. <Experimental conditions> buffer, 50 mM sodium phosphate at pH 7; applied voltage, 30 kV; temperature, 25 °C; injection, 4 mbar 4s; detection wavelength, 214 nm.

(R) is 80% ACN while it is 92% for the best separation efficiency (N) as summarized in Table 1.

In view of optimization for separation resolution, two independent effects intermingled should be simultaneously considered: optimizing mass transfer kinetics and optimizing (maximizing) the difference of thermodynamic analyte-MIP interactions between the two enantiomers. Decreasing the viscosity of eluent by increasing the ACN content is the method of maximizing the mass transfer kinetics (minimizing bandwidths), thus ACN content may be increased as high as possible (92% in this study) so long as the eluent can dissolve a minimum amount of necessary electrolytes if only this effect is considered. However, maximizing differential thermodynamic property (chiral recognition) should also be considered to get the final optimized chromatographic resolution. This effect is very complicated and related to many factors such as relative contributions of specific interactions such as hydrogen bond, electrostatic interaction, hydrophobic interaction, and steric interaction, and MIP cavity structural variation owing to swelling or shrinking, *etc*. Increasing the ACN composition causes increased specific interaction, decreased hydrophobic interaction, and increased MIP swelling while decreasing the ACN composition causes the contrary results. It should be noted that not only too strong interactions or swelling but also too weak interactions or swelling should be avoided to achieve good chiral recognition.[25-34] Thus there should be always an optimized ACN composition, and it should be determined experimentally for each MIP system. The optimized ACN composition of this study was found 80%.

Table 1. The data of number of theoretical plates (N/m) and chromatographic resolution (R), obtained with the (4*S*,5*R*)-4-Methyl-5-phenyl-2-oxazolidinone imprinted MIP capillary column in various ACN compositions[a]

ACN %	N_1[b]	N_2[c]	R
70	36200 ± 1500	30100 ± 1600	2.5 ± 0.15
80	45300 ± 2100	35100 ± 1900	5.0 ± 0.25
92	99000 ± 4600	31800 ± 1800	3.8 ± 0.21

[a]Average and standard deviation based on three batches of columns. [b]Number of theoretical plates for (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone. [c]Number of theoretical plates for (4*R*,5*S*)-4-methyl-5-phenyl-2-oxazolidinone.

Comparative Discussion in View of Previous Studies. The data of chromatographic performances of the MIP of this study in various eluents are assembled in Table 1. The chiral separation performance of (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone may be good enough, but it is clearly inferior to those obtained for MIPs with template of profen drugs[25,27,29] where R values were easily over 5, with N values close to or even higher than 500,000. Profen drugs are 2-arylpropionic acids with a large substituent on the phenyl ring. The unambiguous explanation should begin with the morphology of the (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone MIP layer shown in Figure 1. According to the previous studies,[25-32] the MIP layer should be rugged and porous to give superior separation performance owing to fast mass transfer and high stationary phase surface area. The morphology of the (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone MIP layer shown in Figure 1 is not porous, nor rugged enough.

The reason for relatively inferior morphology of the MIP of this study may be explained through the conclusion of previous studies[27-32] that for good chiral recognition, the chiral center should be accompanied by enough functional groups to enable at least three different interactions, and the two substituents at the chiral center carbon should be hydrogen and methyl group as in profen drugs to ensure superior chiral susceptibility by minimizing spatial congestion near the chiral center. Profen drugs are all with one or more hydrogen bond functional groups and one or more substituted aromatic rings enabling at least one hydrogen bonding, π-π interaction, and steric hindrance interaction. The structure of 4-methyl-5-phenyl-2-oxazolidinone (Fig. 2) lacks some of such characteristics. First of all, it has a plain phenyl group yielding reduced π-π and steric interactions compared to profen drugs having a large substituted aromatic ring. Second, the residual functional groups are in a closed ring. The chiral center carbon is in the ring, and is connected to the phenyl group, thus the chiral center is regarded to be crowded with three large groups. Third, there are two adjacent chiral centers located in the ring, and the two

enantiomers are *S*, *R*-, and *R*, *S*-isomers as shown in Figure 2. Such a format may contribute to loosening of chiral recognition susceptibility. Nevertheless, the chiral separation performance of the MIP of this study is comparable or better than those of general MIPs reported by other groups in the literature.

We have experienced some difficulties to find neutral templates with good chiral recognition susceptibility. It seems hard to obtain commercial template molecules without free amino or carboxylic groups that perfectly fulfill the three points interaction rule. Preparation and validation of more MIP OT-MIP columns are necessary to ensure the applicability of the generalized preparation protocol to neutral templates. Such study is under way with comprehensive search for useful neutral templates.

Conclusion

The (4*S*,5*R*)-4-methyl-5-phenyl-2-oxazolidinone MIP OT-CEC column has been successfully prepared by the pre-established generalized preparation protocol as the MIP with a genuine neutral template. The optimized eluent for the best chromatographic resolution was found 80/20 (v/v %) ACN/ 50 mM sodium phosphate pH 7. The chromatographic resolution at the optimized condition was as high as 5.0. This is the very first report to demonstrate somewhat successful application of the generalized preparation protocol to a MIP with a genuine neutral template although further work with more neutral templates are required in the future.

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