Molecularly imprinted uniform-size polymer-based stationary phase for high-performance liquid chromatography
Structural contribution of cross-linked polymer network on specific molecular recognition

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Abstract

Non-covalently molecularly imprinted, uniform-size, polymer-based, stationary phases were prepared by a two-step swelling technique using isomers of diaminonaphthalene or a chiral amide derived from (S)-α-methylbenzylamine as the template molecule. Methacrylic acid worked as an effective host molecule for diaminonaphthalene templates, however, an imprinted, cross-linked, polymer-based, stationary phase without such a relatively strong host functionality unexpectedly showed moderate molecular recognition, which suggested that the cross-linked polymer network could memorize the shape of a template. In chiral separation of amide derivatives, a cross-linked polymer network also memorized the shape of a chiral template, resulting in chiral resolution. In addition, a chiral cross-linking agent having similar functionality to the chiral amide template could enhance molecular recognition drastically. Further studies suggested that this enhancement in chiral recognition was due to a favorable structural interaction within the specific recognition sites.

Keywords: Molecular imprinting; Stationary phases, LC; Enantiomer separation; Chiral stationary phases, LC; Diaminonaphthalenes; Methylbenzylamines; Phenylglycinols

1. Introduction

The molecular imprinting technique is an effective strategy to prepare stationary phases having a specific molecular recognition [1–3]. In this technique, a template molecule is admixed with monomers to afford polymeric separation media after their polymerization. The template molecule usually involves relatively polar functional groups [4,5], such as carboxylic acid, hydroxyl, amino, and/or aromatic groups, and therefore appropriate host monomers having functional groups which may interact with the functional groups of the template molecule through intermolecular interactions such as ionic interaction are often utilized to compose a more effective imprinted site [6].

Usually, non-aqueous bulk polymerization methods [4] including a preparation method for continuous rod-type separation media [7] are utilized to obtain molecularly imprinted separation media, although molecularly imprinted polymer prepared by

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the bulk polymerization method should be grained and sieved to produce packing material [8]. This is because the normal (O/W) suspension polymerization method to produce spherical polymer beads requires an aqueous suspension medium, and the water is thought to weaken the interaction between the template molecule and its host molecule due to high polarity of the water medium adjacent to oil droplets of the monomer mixture. Recently, a highly selective, in situ prepared, imprinted material was prepared using aqueous porogen [9]. In this paper, the author also mentioned weak or moderate templates which required a low polymerization temperature and exclusion of water porogen during imprinting in order to produce the recognition effect.

Recently, we reported molecularly imprinted, uniform-size, polymer-based, stationary phases for high-performance liquid chromatography (HPLC) using an isomer of diaminonaphthalene as the template [10]. Although we applied a typical two-step swelling and polymerization method [11] in which water was required as the suspension medium, the prepared stationary phase showed an equivalent molecular recognition to that with the previously reported, continuous, rod-type, polymeric separation medium prepared by a kind of non-aqueous bulk polymerization [12]. This finding suggests that molecularly imprinted, spherical, polymer-based separation media can be prepared in a usual suspension polymerization system without loss of molecular recognition.

In our previous report [10], methacrylic acid was utilized as a host molecule to the isomer of diaminonaphthalene [12] in the expectation of possible interaction between the amino group and carboxylic acid of the host monomer. Actually, the interaction played an important role in isomer separation, while severe peak tailing for amines was found due to the relatively strong ionic interaction between the amino group and carboxylic acid. This interaction also affects retention of other solutes with amino functionality, especially with uniform-size, polymeric separation media because this method requires an aqueous polymerization medium, as mentioned before, and thermal initiation of polymerization.

In this paper, we wish to describe the preparation and chromatographic properties of molecularly imprinted, uniform-size, polymer-based, stationary phases through investigation of the effect of a monofunctional host molecule on basic separation selectivity and the effect of a chiral cross-linking agent on molecular recognition ability.

2. Experimental section

2.1. Materials

Ethylene dimethacrylate was purchased from Tokyo Chemical Industry (Tokyo, Japan), while methacrylic acid was purchased from Nacalai Tesque (Kyoto, Japan). Both monomers were purified by general distillation techniques in vacuo to remove the polymerization inhibitor. Benzoyl peroxide or α,α'-azobisisobutyronitrile as the radical initiator was purchased from Nacalai Tesque and utilized as received.

1,5- and 1,8-diaminonaphthalenes and (S)-$(-)$-α-methylbenzylamine were purchased from Nacalai Tesque, while (S)-$(+)$-N-(3,5-dinitrobenzoyl)-α-methylbenzylamine and its antipode as well as other chiral solutes mentioned in Section 3.2 were purchased from Aldrich Chemical (Milwaukee, USA). S-$(+)$-α-Phenylglycinol and R-$(-)$-α-phenylglycinol were purchased from Fluka Chemika, Japan (Tokyo, Japan).

Chiral cross-linking agents were prepared using the chiral phenylglycinols through a typical condensation reaction with methacryloly chloride (Aldrich) and purified using column chromatography on silica gel in a mixture of hexane and ethyl acetate (3:1, v/v).

2.2. Two-step swelling and polymerization method

Uniformly sized, polystyrene seed particles utilized as the shape template were prepared by an emulsifier-free emulsion polymerization and purified by a previously reported method [13]. The size of the seed particle was ca. 1 μm in diameter.

Preparation of uniformly sized, macroporous, molecularly imprinted polymer beads as well as non-imprinted polymer beads by a two-step swelling and polymerization method was carried out as follows. A water dispersion of the uniformly sized, polystyrene seed particles (9.5×10^{-2} g/ml), 1.4 ml, was admix-

ed with a micro-emulsion prepared from 0.95 ml of dibutyl phthalate as activating solvent [14], 0.085 g of benzoyl peroxide or α,α'-azobisisobutyronitrile, 0.04 g of sodium dodecyl sulphate, and 10 ml of distilled water by sonication. This first-step swelling was carried out at room temperature with stirring at 125 rpm. Completion of the first-step swelling was determined by the point of vanishing of the micro oil droplets in the added micro-emulsion using an optical microscope.

A dispersion of 10 ml of cross-linking agent [or 95:5 (weight ratio) of a mixture of ethylene dimethacrylate and host or additive] and 10 ml of toluene or cyclohexanol as porogenic solvent into 90 ml of water containing 1.92 g of polyvinylalcohol (dp=500, saponification value=86.5 to 89 mol%) as a dispersion stabilizer was added to the dispersion of swollen particles. The swelling was carried out at room temperature for 12 h with stirring at 125 rpm. When the template molecule was added, 1 g of the template was admixed with the monomers utilized to prepare the dispersion for the second-step swelling.

After the second-step swelling was completed, the polymerization procedure was started at 70°C under argon atmosphere with slow stirring. After 24 h, the dispersion of polymerized beads was poured into 250 ml of water to remove the suspension stabilizer (polyvinylalcohol), and the supernatant was discarded after sedimentation of the beads.

The polymer beads were redispersed into methanol, and the supernatant was again discarded after sedimentation. This procedure was repeated three times in methanol and twice in tetrahydrofuran (THF), then the polymer beads were filtered on a membrane filter and washed with THF and acetone followed by drying at room temperature to determine the chemical yields of the beads. The final bead size was 5.6 μm, for which the calculated value was 6.0 μm.

The chemical yields of all particles prepared by polymerization were higher than 88%, and non-covalently involved template molecules were found to be completely removed from the polymer particle by the washing procedure, as certified by elemental analysis data. For example, with 1,8-diaminonaphthalene as template, C%, H% and N% of the particle with methacrylic acid were 58.54%, 6.98%, and 0.00%, respectively, while those without

the template were 58.91%, 7.00%, and 0.00%, respectively. For a chiral template, C%, H%, and N% of the imprinted particles were 59.52%, 6.98%, and 0.00%, respectively, while those of the base particles were 58.31%, 7.00%, 0.00%, respectively, where the calculated values are 60.59%, 7.12%, and 0.00%, respectively.

The prepared beads were packed into a stainless-steel column (4.6 mm I.D.×150 mm) by a slurry technique using aqueous acetonitrile as the slurry medium to evaluate their chromatographic characteristics. Void markers used were acetone in acetonitrile mobile phase and in normal-phase mode, both of di-*tert.*-butylbenzene and *n*-pentylbenzene.

2.3. Chromatography

All the chromatographic solvents were purchased from Nacalai Tesque and used as received. HPLC was performed with a Jasco 880-PU Intelligent HPLC Pump equipped with a Rheodyne 7125 valve loop injector and a Waters Model 440 UV detector set at 254 nm. Chromatography was carried out at 30±0.5°C and a Shimadzu C-R4A was utilized as recorder.

3. Results and discussion

3.1. Diaminonaphthalene as template

As we reported in the previous paper [10], the isomer of diaminonaphthalene utilized as template is eluted after the other isomer on each imprinted stationary phase in 100% acetonitrile as the mobile phase (Fig. 1). On these stationary phases, methacrylic acid was combined as host molecule to the template diaminonaphthalene. On both chromatograms, the more hydrophobic naphthalene is found to be eluted much faster than both 1,5- and 1,8-diaminonaphthalene, even in reversed-phase mode. This finding supports the hypothesis that combined methacrylic acid plays an important role in retaining the diaminonaphthalenes through the interaction between the acid and the amino groups.

This contribution of methacrylic acid functionality to favorable retention with the amino group is also
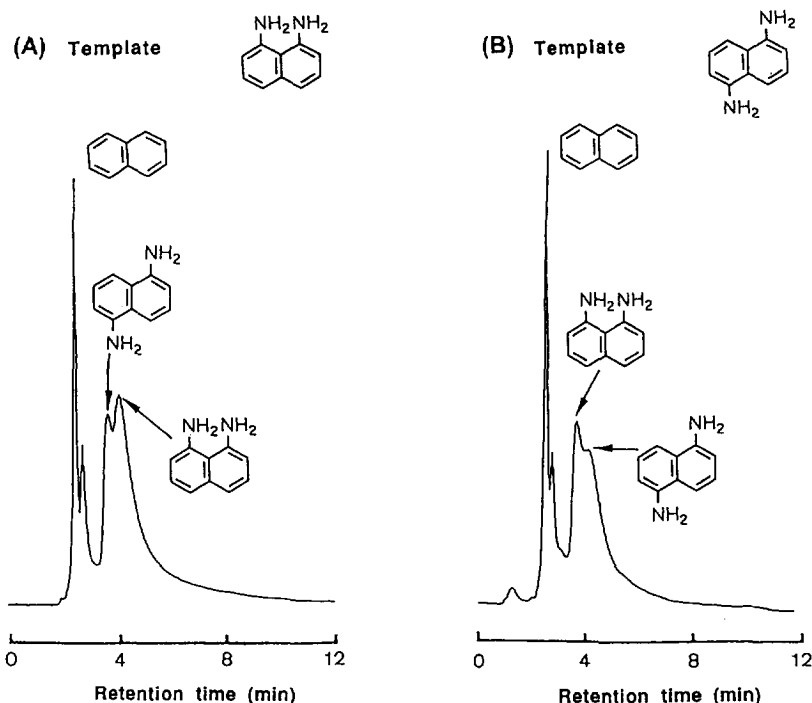
Fig. 1. Separations of isomers of diaminonaphthalene on moleculary imprinted stationary phases. Chromatographic conditions: mobile phase, 100% acetonitrile; flow-rate, 1.0 ml/min; detection, UV 254 nm; column size, 4.6 mm I.D.×150 mm. Solutes, 1,5-diaminonaphthalene (0.3 μg), 1,8-diaminonaphthalene (0.3 μg).

found for other aromatic amines, as summarized in Table 1. Aniline is eluted after benzene, with a broader peak shape, and furthermore, pyridine affords the largest k' value in all the solutes tested.

Addition of 0.1% triethylamine to the mobile phase does not change the k' value of benzene, while other amine compounds including diaminonaphthalenes are eluted much faster than in 100% aceto-

nitrile as the mobile phase. Since triethylamine would competitively block the methacrylic acid functionality on the stationary phase, these findings support the role of methacrylic acid functionality in retentivity as mentioned above. The effect also confirms earlier results using buffered mobile phases [15].

Methacrylic acid is thus one of the important

Table 1
Effect of triethylamine on separation and retentivity

Template[a]	Mobile phase[b]	$k'(1,5-)$[c]	$k'(1,8-)$[d]	$\alpha(k'_{1,5-}/k'_{1,8-})$	k'(pyridine)	k'(aniline)	k'(benzene)
1,5-	AN	1.46	0.94	1.55	1.51	0.40	0.11
1,5-	AN+TEA	0.99	0.72	1.38	0.79	0.30	0.12
1,8-	AN	0.93	1.18	0.79	1.23	0.34	0.11
1,8-	AN+TEA	0.69	0.82	0.84	0.70	0.28	0.12

[a] Diaminonaphthalene used as template; 1,5-: 1,5-diaminonaphthalene; 1,8-: 1,8-diaminonaphthalene.
[b] AN: 100% acetonitrile; AN+TEA: 100% acetonitrile (0.1% triethylamine).
[c] k' of 1,5-diaminonaphthalene.
[d] k' of 1,8-diaminonaphthalene.
Chromatographic conditions: flow-rate, 1 ml/min: detection, UV 254 nm.

factors in the molecular imprinting technique which aid separation between the template and its isomer with larger retention, however, other solutes having the same functionality as the template such as pyridine are also affected, resulting in greater retention with a broader peak shape which tends to have a possibility of peak-overlapping with the template molecule during analysis of those mixed solutes. In other words, strong host functionalities often afford even worse selectivity in the retentivity between solute having the same functionality and the solute used as template.

Although the retention contributed by the methacrylic acid functionality is blocked by the addition of triethylamine in the mobile phase, each diaminonaphthalene used as template is still retained longer than the isomer on the corresponding imprinted stationary phase. Since the base stationary phase prepared without the template molecule cannot separate the diaminonaphthalenes, the observed moderate molecular recognition can only be explained based on a contribution of the recognition site formed on the cross-linked polymer network.

In order to investigate the above findings concerning the contribution of a cross-linked polymer network, we prepared an imprinted stationary phase, but methacrylic acid was not utilized. The results on a stationary phase using 1,8-diaminonaphthalene as template as well as a non-imprinted base stationary phase are summarized in Table 2. The retention time of either diaminonaphthalene is found to be shorter on the stationary phase prepared without the host when compared to the stationary phase prepared with the host, while larger k' values are obtained than those on the non-imprinted base stationary phase prepared without the host monomer.

Although the base stationary phase cannot separate the isomers of diaminonaphthalene, the imprinted stationary phase without the host monomer, methacrylic acid, can separate the isomer as the template isomer is retained longer. Interestingly, pyridine is eluted much faster on both the base stationary phase and the imprinted stationary phase prepared without the host. These differences in k' values of pyridine support the contribution of methacrylic acid functionality to the retentivity of amino functionality.

The addition of triethylamine to the mobile phase for the non-imprinted base stationary phase does not affect the k' values of all the solutes tested. This finding implies that the interaction between ethylene dimethacrylate and amino functionality is relatively weak, and the observed molecular recognition on the imprinted stationary phase prepared without the host monomer is due to the recognition site for the shape of the template on the cross-linked polymer network.

Although the addition of triethylamine affords limited recognition of diaminonaphthalene, this recognition varies inversely to the observed molecular recognition on the imprinted stationary phase, thus it is almost negligible. Since the chemical composition of both stationary phases is absolutely identical, the observed molecular recognition suggests that some specific site for the molecular recognition is formed on the cross-linked structure, as expected from discussions in the previous paragraph.

Table 2
Retentivity on imprinted particle prepared without the host monomer

Template	Host	$k'(1,8-)$[a]	$k'(1,5-)$[b]	$\alpha(k'_{1,8-}/k'_{1,5-})$	k'(pyridine)
None	None	0.46	0.46	1.00	0.15
1,8-[c]	None	0.66	0.60	1.10	0.14
1,8-[c]	methacrylic acid	1.18	0.93	1.27	1.23
None	None	0.50[d]	0.47[d]	0.94	0.15[d]

[a] k' of 1,8-diaminonaphthalene.
[b] k' of 1,5-diaminonaphthalene.
[c] 1,8-diaminonaphthalene.
[d] 0.1% triethylamine was added to the mobile phase.
Mobile phase: 100% acetonitrile. Flow-rate: 1.0 ml/min. Detection: UV 254 nm.

3.2. (S)-(+)-N-(3,5-dinitrobenzoyl)-α-methyl-benzylamine as template

Chiral resolution is a good target of the molecular imprinting technique. Many chiral solutes such as drugs have been reported to be recognized or separated with molecularly imprinted separation media [16–18]. The template we selected for the study is (S)-(+)-N-(3,5-dinitrobenzoyl)-α-methylbenzyl-amine, (S)-DNB (Fig. 2). Although non-derived α-methylbenzylamine can be used as a template and should be separated by a molecularly imprinted stationary phase using methacrylic acid as a host molecule, here the amide derived from α-methyl-benzylamine is employed to ascertain the structural contribution of a cross-linked polymer network as discussed in the previous section, using diaminonaphthalene as the template.

As summarized in Table 3, the non-imprinted base stationary phase prepared using only ethylene di-

methacrylate as the cross-linking agent cannot resolve the enantiomers of three kinds of chiral amides employed as solutes. When (S)-DNB is utilized as the template, molecular recognition of the template molecule allows chiral resolution with larger retention times. Although the k' values of the template and the antipode increase by 73% and 34%, respectively, that of N-(4-nitrobenzoyl)-α-methylbenzyl-amine NB, with a similar structure to the template, grows by 11%, while that of N-benzoyl-α-methyl-benzylamine B is only 7% larger without resolution.

As determined in the previous section, the imprinted stationary phases prepared without methacrylic acid only afforded larger k' values for the template molecule and its isomer when compared to the non-imprinted base stationary phase. Here, the imprinted stationary phase with the cross-linking agent ethylene dimethacrylate again affords chiral resolution with larger k' values to the template and the antipode in comparison with those on NB or B.
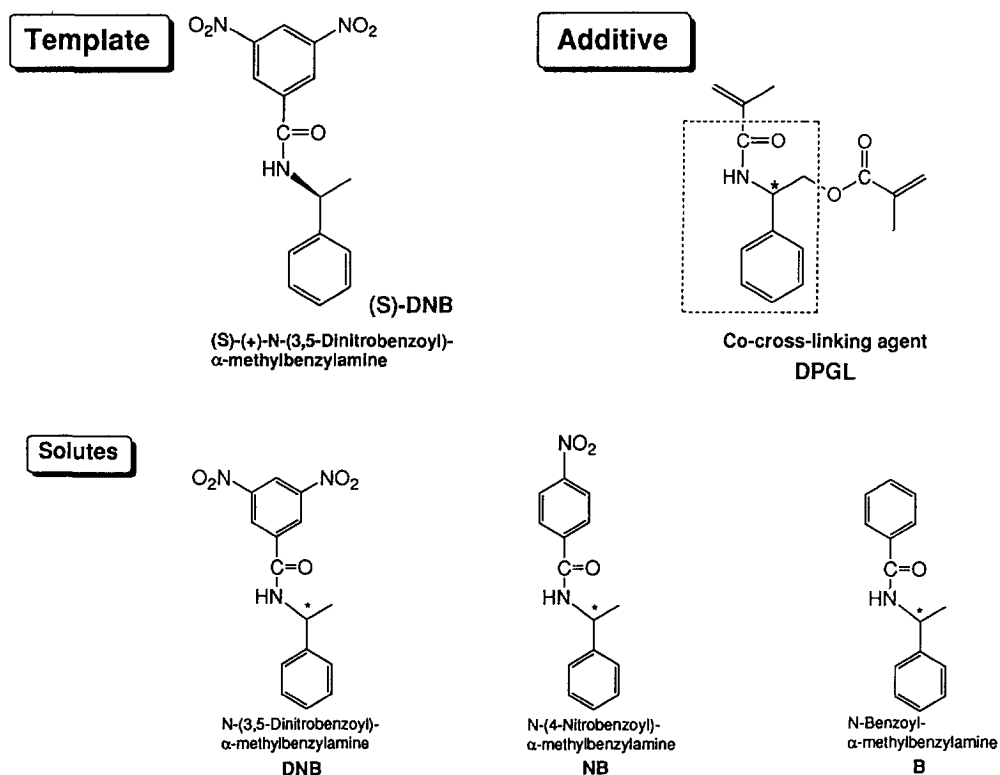

Fig. 2. Chiral template, additive, and solutes and their abbreviations.

Table 3
Separation of chiral amide derivatives[a]

Template	Additive	(R)-DNB k'	(S)-DNB k'	α	(R)-NB k'	(S)-NB k'	α	(R)-B k'	(S)-B k'	α
No	No	1.73	1.73	1.01	1.63	1.63	1.02	1.21	1.21	1.00
(S)-DNB	No	2.32	3.00	1.29	1.82	1.82	1.02	1.30	1.30	1.01
(S)-DNB	(R)-DPGL	2.26	4.88	1.91	1.77	1.94	1.09	1.25	1.31	1.05
(S)-DNB	(±)-DPGL	2.55	3.69	1.44	1.86	1.91	1.02	1.31	1.31	1.00
(S)-DNB	(S)-DPGL	2.41	3.00	1.24	1.75	1.70	0.97	1.19	1.18	0.99

[a]See for abbreviations Fig. 2. Chromatographic conditions are the same as those in Fig. 3.

This suggests that the cross-linked polymer network can memorize the shape of the chiral template specifically.

In order to enhance the specific molecular recognition within the imprinted polymer network, a chiral cross-linking agent, N,O-dimethacryloyl phenylglycinol, DPGL (Fig. 2), was selected. This chiral cross-linking agent is easy to prepare and involves amide functionality, which may lead to a favorable molecular interaction through a possible amide–amide interaction with the template. In addition, as reported in the previous paper dealing with uniform-size, polymer-based, chiral separation media with poly-methacrylamide as the chiral selector [19], this chiral cross-linking agent did not show any effective chiral resolution when the usual co-polymerization technique was utilized for preparation of the chiral stationary phase with the cross-linking agent. This means that the chiral cross-linking agent DPGL does not work as a chiral selector on normal chiral stationary phases prepared by the co-polymerization technique. A similar poor recognition ability was also reported previously using a similar chiral cross-linking agent [20]. Therefore, we utilized here (R)-DPGL and its antipode (S)-DPGL as well as racemic DPGL as co-cross-linking agents.

(R)-DPGL has the same chirality as the template and is found to enhance the specific molecular recognition; the largest α value is afforded to the chiral template (Fig. 3). Interestingly, the k' value of the solute used as the template becomes much larger than that on the imprinted stationary phase prepared without the chiral cross-linking agent, while k' values of the antipode as well as other solutes are almost equivalent (Table 3). Although the chiral resolution of DNB is drastically affected by the addition of (R)-DPGL, almost no separation occurs

for similar solutes, NB and B. This is because (R)-DPGL itself allows low chiral recognition, as mentioned before.

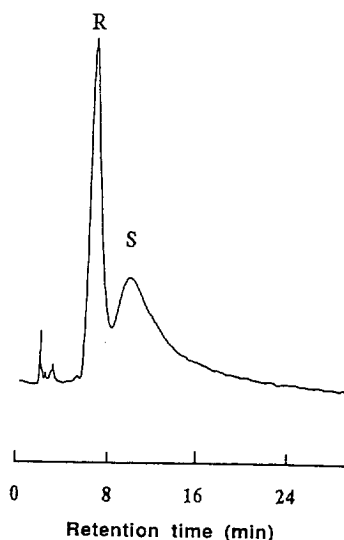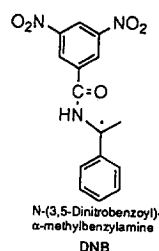If the chiral selectivity of the added chiral cross-linking agent is the dominant factor in the enhance-

O₂N

NO₂

C=O

HN

N-(3,5-Dinitrobenzoyl)-
α-methylbenzylamine
DNB

Fig. 3. Resolution of DNB on the imprinted stationary phase prepared with (R)-DPGL. Chromatographics conditions: mobile phase, hexane–ethyl acetate (1:1, v/v); flow-rate, 1.0 ml/min; detection, UV 254 nm; column size, 4.6 mm I.D.×150 mm.

ment of the specific molecular recognition to the template molecule, addition of racemic DPGL should annul the enhancement of specific molecular recognition on the imprinted stationary phase. However, the imprinted stationary phase shows intermediate molecular recognition between the stationary phases prepared with and without (R)-DPGL. In addition, the antipode of (R)-DPGL is found to afford quite similar molecular recognition to the imprinted stationary phase prepared without DPGL. This implies that (S)-DPGL does not disturb the chiral recognition observed with the imprinted stationary phase prepared without any chiral cross-linking agents (DPGL).

Addition of racemic or (S)-DPGL does not change the k' values of other solutes as found with the imprinted stationary phase with (R)-DPGL either, while very limited inversion of chiral recognition is observed with the stationary phase prepared with (S)-DPGL. This is probably due to the inverse chirality of the cross-linking agent, but as mentioned before, the chiral recognition of DPGL is not high [18], and thus the inversion of chiral recognition is almost negligible.

These findings strongly suggest that only a favorable interaction between solutes and the combined chiral cross-linking agent within the imprinted site enhances specific molecular recognition toward the solute used as the template with specific enhancement of the retention on the template. This also suggests that the relatively weak intermolecular interaction can be enhanced within the specific recognition site where the template molecule fits well.

4. Conclusion

This work still includes speculative explanations, but the molecularly imprinted stationary phase prepared without a host monomer which has a relatively strong interaction with the template molecule affords moderate molecular recognition because the cross-linked polymer network memorizes the shape of the template utilized. The host monomer plays an important role in promoting specific molecular recognition between isomers utilized as the template, but other solutes having similar functionality to the

template are also affected by the host functionality, resulting in a non-specific enhancement of retention. On the other hand, the cross-linked, molecularly imprinted, stationary phase prepared without a strong host monomer can enhance only retention of the solute used as the template because of the relatively weak intermolecular interactions; thus, specificity among the solutes having similar functionality is also obtained. Addition of the favorable chiral cross-linking agent enhances this specific molecular recognition without loss of the solute specificity. Further study using the chiral cross-linking agent is in progress.

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