mobile phase additives. This means that all chiral selectors covalently bound to supports can be used for the separation of enantiomers by addition to the mobile phase. With chiral mobile phase additive techniques, there are at least two possible mechanisms; one is that the chiral mobile phase additive and the enantiomers may form diastereomers in the mobile phase. Another is that the stationary phase may be coated with the chiral mobile phase additive, resulting in diastereomeric interactions with the enantiomeric pairs during chromatography. It is thought that both mechanisms occur depending on both the stationary and mobile phases used.

The techniques using chiral mobile phase additives can be divided into three categories: metal complexation used in chiral ligand exchange chromatography, the use of various uncharged additives, and the ion-pairing techniques used for charged analytes. **Table 3** shows chiral selectors used as mobile phase additives.

See also: **II/Chromatography: Liquid:** Derivatization; Mechanisms: Chiral; **III/Chiral Separations:** Cellulose and Cellulose Derived Phases; Chiral Derivatization; Cyclodextrins and Other Inclusion Complexation Approaches; Ligand Exchange Chromatography; Ion-Pair Chromatography; Protein Stationary Phases; Synthetic Multiple Interaction ('Pirkle') Stationary Phases. **Inclusion Complexation: Liquid Chromatography.**

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Molecular Imprints as Stationary Phases

M. Kempe, Lund University, Lund, Sweden

In 1949, Frank Dickey published what can be considered the first paper on a molecularly imprinted synthetic material. The work was inspired by the theories of Dickey's mentor Linus Pauling, who had suggested that the primary structures of all polypeptides constituting the antibodies are the same and that the diversity originates from folding directed by physical contact with the antigens. Even if Pauling's hypothesis on antibodies later turned out to be incorrect, his ideas laid the foundation for the concept of molecular imprinting. Consistent with these early studies, molecular imprinting can be defined as a method in which the selectivity of a material for a chosen molecule is induced by the presence of the molecule during the preparation, assembling or rearrangement of the material.

Dickey's studies were followed by several other investigations in the same direction, but it was not until the 1970s that the field of molecular imprinting started to mature to its present form. Wulff introduced a new approach that allowed the introduction of functional groups at defined positions in synthetic network polymers. This approach is often referred to as covalent molecular imprinting, to distinguish it from the noncovalent approach developed by Mosbach and his co-workers in the early 1980s.

The field of molecular imprinting is growing rapidly. Molecularly imprinted polymers (MIPs) have found application as stationary phases for chiral separations and solid-phase extractions, as synthetic antibodies in competitive ligand-binding assays, as recognition elements in sensors and as catalysts of chemical reactions. The concepts of molecular imprinting will be described briefly here. For a more detailed description of the imprinting principle and the preparation of molecularly imprinted polymers, see 'Affinity Separation: Imprint Polymers' in this encylopedia. The remainder of this paper focuses on the use of molecularly imprinted polymers as chiral stationary phases (CSPs).

The Concepts of Molecular Imprinting

Molecular imprinting, sometimes referred to as template polymerization, is a technique for preparing synthetic polymers of predetermined selectivity. Receptor-like binding sites are tailor-made *in situ* by the copolymerization of cross-linkers and functional monomers, which are interacting covalently or noncovalently with print molecules (or templates). After polymerization, the print molecules are removed from the polymer, either by extraction or by chemical cleavage, leaving recognition sites complementary to the print molecules in the shape and positioning of functional groups. The polymer is subsequently able to rebind the print molecules. The noncovalent approach of molecular imprinting is exemplified in **Figure 1**.

The association/dissociation kinetics of noncovalent MIPs is in general faster than is observed with polymers prepared by the covalent approach. For this reason, the former polymers are more attractive as stationary phases for chromatography. Even if several examples of covalently imprinted stationary phases have been reported, it was not until the development of the noncovalent approach that the technique became competitive for the preparation of CSPs. This review will therefore focus on noncovalent molecular imprinting. The covalent approach has been covered in several excellent reviews by Wulff.

Even if the general understanding has been that the selectivity of MIPs is due to the formation of specific recognition sites complementary to the print molecules, early critics of the technique argued that the recognition could come from binding to print molecules entrapped in the highly cross-linked polymers. A small percentage of the print molecules are inaccessible and remain in the polymer network after extraction (normally less than 1%). However, this was ruled out as the cause of the selectivity by experiments showing that a polymer containing covalently bound print molecules did not exhibit any selective binding of the print molecule.

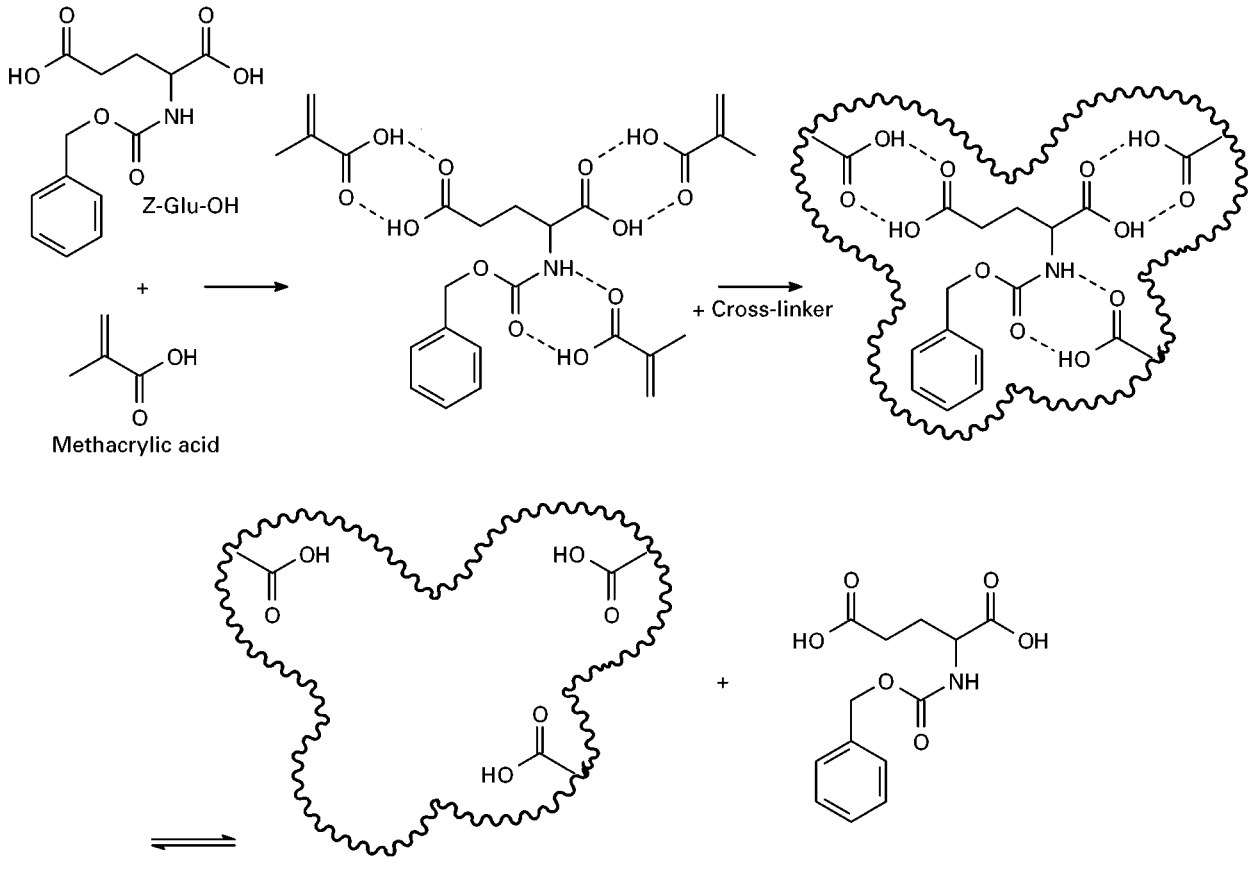


Figure 1 Schematic representation of the concept of noncovalent molecular imprinting. Functional monomers, in this case methacrylic acid, interact with the print molecule, Z-Glu-OH. Cross-linker is added and the polymerization is initiated. The interactions are maintained in the resulting polymer. The print molecule is removed from the polymer by extraction, leaving a specific recognition site complementary to the print molecule in shape and positioning of the functional groups. The polymer has attained a memory of the print molecule and is able selectively to rebind it.

Molecular imprinting produce recognition sites with a distribution of binding strengths; the sites are heterogeneous. Some sites have a highly selective affinity for the template, whereas others are less selective. When used for chromatographic applications, the heterogeneity is reflected in band broadening and asymmetric peaks.

Chiral Separations

Initial studies on noncovalent MIPs focused mainly on the preparation of materials selective for amino acid derivatives. The polymers possessed not only a selectivity for the amino acid used as the print molecule, but were also found to be enantioselective; the enantiomer present during the polymerization was preferentially bound. These findings, together with observations that the polymers were mechanically and chemically stable, made them attractive as CSPs and spurred further research.

The imprinting effects of MIPs prepared with optically active compounds as the print molecules are readily demonstrated by chromatographic evaluation. For example, when the L-enantiomer of an amino acid derivative is used as the print species, a column packed with the resulting polymer will retain the L-enantiomer longer than the D-enantiomer, and vice versa when the D-enantiomer is used as the print molecule. Reference polymers prepared with the racemate or without print molecule will not be able to resolve the racemate. A stereoselective memory is hence induced in the polymers by the print molecules.

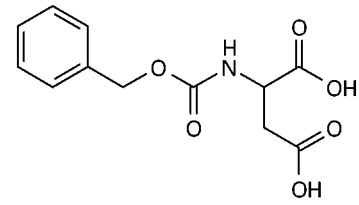
Molecularly imprinted CSPs have been prepared for applications in high performance liquid chromatography (HPLC), thin-layer chromatography (TLC), capillary electrophoresis (CE) and capillary electrochromatography (CEC). CSPs for HPLC are by far the most studied.

HPLC

A large number of chiral amino acids and peptides have been imprinted. Several MIPs selective for pharmaceuticals have also been described. The most widely used method has been bulk polymerization followed by grinding, sieving and packing into HPLC columns. Alternatively, the polymers can be prepared by any of the methods discussed in 'Affinity Separation: Imprint Polymers' in this encyclopedia. Some examples of MIP CSPs are shown in **Table 1**.

The selectivities are in many cases comparable to those of commercially available CSPs. For example, a separation factor (α) of 17.8 was found for the separation of the two enantiomers of a dipeptide on *poly*(methacrylic acid-*co*-EDMA) (EDMA = ethylene glycol dimethacrylate) imprinted with one of the enantiomers (**Figure 2**).

The specificity and selectivity of MIPs can be fine-tuned by careful choice of monomers and solvent, and by optimizing the molar ratios of the components in the polymerization mixture. The recognition relies on multiple interaction points. The more funtionalized the print molecule is, the more interactions are possible. An example of a highly selective polymer is *poly*(4-vinylpyridine-*co*-EDMA) imprinted with Z-L-aspartic acid (**1**). The chromatogram in **Figure 3**A shows the separation of racemic Z-aspartic acid on this CSP. Aspartic acid and glutamic acid differ by only one methylene unit, but despite this small difference, the Z-aspartic acid-imprinted CSP was not able to resolve racemic Z-glutamic acid (**2**) (**Figure 3**B). The side chain of Z-L-glutamic acid cannot be accommodated into the recognition site in a way that allows specific interaction between the carboxy functionality and the positioned pyridine group of the polymer. The same type of polymer, imprinted with Z-L-glutamic acid, was able to resolve racemic Z-glutamic acid, but not racemic Z-aspartic acid. Similar observations have been done on *poly*(methacrylic acid-*co*-EDMA) imprinted with these print molecules.



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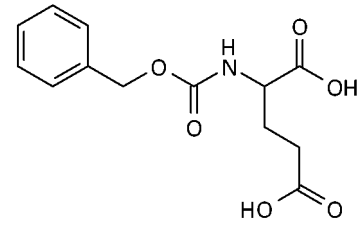

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Polymers imprinted with Z-L-phenylalanine (**3a**) were able to separate racemic Z-phenylalanine efficiently (**Table 2**). Racemic Z-alanine (**3b**) could also be separated on these CSPs, even if lower separation factors were observed. When the amino-group of the racemate was protected with *tert*-butyloxycarbonyl (Boc) (**3c**) or 9-fluorenylmethyloxycarbonyl (Fmoc) (**3d**), the separations were poorer than with the racemate of the print molecule, which was protected with benzyloxycarbonyl (Z). In contrast to the CSPs described above, which were unable to separate the racemate of a molecule which only differed from the

Table 1 A selection of molecularly imprinted CSPs for HPLC[a]

Print molecule	*Polymer*[b]	α[c]	R_S[d]	*f*/*g*[e]
Amino acids				
H-L-Phe-OH[f]	*poly*(CuVBIDA-*co*-EDMA)	1.45	n.d.	n.d.
H-L-Phe-NHPh[g]	*poly*(MAA-*co*-EDMA)	13	n.d.	n.d.
H-D-*p*-NH_2Phe-NHPh[h]	*poly*(MAA-*co*-EDMA)	15	n.d.	n.d.
Ac-D-Trp-OMe[i]	*poly*(MAA-*co*-EDMA)	3.92	2.2	1.0
Ac-L-Trp-OH[j]	*poly*(AA-*co*-EDMA)	3.24	2.02	n.d.
Boc-L-Trp-OH[i]	*poly*(MAA-*co*-2VPy-*co*-EDMA)	4.35	1.9	1.0
Fmoc-L-Phe-OH[k]	*poly*(MAA-*co*-EDMA)	1.36	n.d.	n.d.
Z-L-Asp-OH[l]	*poly*(4VPy-*co*-EDMA)	2.81	1.22	0.81
Z-L-Phe-OH[m]	*poly*(MAA-*co*-TRIM)	2.29	3.14	1.00
Z-L-Tyr-OH[m]	*poly*(MAA-*co*-PETRA)	2.86	5.47	1.00
Dansyl-L-Phe-OH[i]	*poly*(MAA-*co*-2VPy-*co*-EDMA)	3.15	1.6	0.96
Small peptides				
H-L-Phe-Gly-NHPh[n]	*poly*(MAA-*co*-EDMA)	5.1	n.d.	n.d.
Boc-L-Phe-Gly-OEt[m]	*poly*(MAA-*co*-TRIM)	3.04	3.44	1.00
Z-L-Ala-L-Ala-OMe[m]	*poly*(MAA-*co*-TRIM)	3.19	4.50	1.00
Ac-L-Phe-L-Trp-OMe[o]	*poly*(MAA-*co*-EDMA)	17.8	n.d.	1.00
Z-L-Ala-Gly-L-Phe-OMe[m]	*poly*(MAA-*co*-TRIM)	3.60	4.15	1.00
Pharmaceuticals				
(*S*)-Timolol[p]	*poly*(MAA-*co*-EDMA)	2.9	2.0	n.d.
(*S*)-Naproxen[q]	*poly*(4VPy-*co*-EDMA)	1.65	n.d.	n.d.
(*S*,*R*)-Ephedrine[r]	*poly*(MAA-*co*-TRIM)	3.42	1.6	n.d.
(*S*,*S*)-Pseudoephedrine[r]	*poly*(MAA-*co*-TRIM)	3.19	1.8	n.d.

[a]The print molecules and their optical antipodes were separated.

[b]AA, Acrylamide; CuVBIDA, Cu(II)[*N*-(4-vinylbenzyl)]iminodiacetate; EDMA, ethylene glycol dimethacrylate; Ita, itaconic acid; MAA, methacrylic acid; PETRA, pentaerythritol triacrylate; TRIM, trimethylolpropane trimethacrylate; 2VPy, 2-vinlypyridine; 4VPy, 4-vinlypyridine.

[c]The separation factor were calculated as $\alpha = k'_{\text{print molecule}}/k'_{\text{optical antipode}}$; $k' = (t - t_0)/t_0$; t is the retention time of the analyte and t_0 is the retention time of unretained compound (the void).

[d]The resolution factors (R_S) were calculated according to Wulff G, Poll HG and Minárik M (1986) Enzyme-analogue built polymers. XIX. Racemic resolution on polymers containing chiral cavities. *Journal of Liquid Chromatography* 9: 385–405.

[e]The resolution factors (f/g) were calculated according to Meyer VR (1987) Some aspects of the preparative separation of enantiomers on chiral stationary phases. *Chromatographia* 24: 639–645.

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[g]Data from Sellergren B and Shea KJ (1993) Chiral ion-exchange chromatography. Correlation between solute retention and a theoretical ion-exchange model using imprinted polymers. *Journal of Chromatography A* 654: 17–28.

[h]Data from Sellergren B and Nilsson KGI (1989) Molecular imprinting by multiple noncovalent host-guest interactions: Synthetic polymers with induced specificity. *Methods in Molecular and Cellular Biology* 1: 59–62.

[i]Data from Ramström O, Andersson LI and Mosbach K (1993) Recognition sites incorporating both pyridinyl and carboxy functionalities prepared by molecular imprinting. *Journal of Organic Chemistry* 58: 7562–7564.

[j]Data from Yu C and Mosbach K (1997) Molecular imprinting utilizing an amide functional group for hydrogen bonding leading to highly efficient polymers. *Journal of Organic Chemistry* 62: 4057–4064.

[k]Data from Kempe M and Mosbach K (1994) Chiral recognition of N^{α}-protected amino acids and derivatives in non-covalently molecularly imprinted polymers. *International Journal of Peptide and Protein Research* 44: 603–606.

[l]Data from Kempe M, Fischer L and Mosbach K (1993) Chiral separation using molecularly imprinted heteroaromatic polymers. *Journal of Molecular Recognition* 6: 25–29.

[m]Data from Kempe M (1996) Antibody-mimicking polymers as chiral stationary phases in HPLC. *Analytical Chemistry* 68: 1948–1953.

[n]Data from Andersson LI, O'Shannessy DJ and Mosbach K (1990) Molecular recognition in synthetic polymers: preparation of chiral stationary phases by molecular imprinting of amino acid amides. *Journal of Chromatography* 513: 167–179.

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[p]Data from Fischer L, Müller R, Ekberg B and Mosbach K (1991) Direct enantioseparation of β-adrenergic blockers using a chiral stationary phase prepared by molecular imprinting. *Journal of the American Chemistry Society* 113: 9358–9360.

[q]Data from Kempe M and Mosbach K (1994) Direct resolution of naproxen on a non-covalently molecularly imprinted chiral stationary phase. *Journal of Chromatography A* 664: 276–279.

[r]Data from Ramström O, Yu C and Mosbach K (1996) Chiral recognition in adrenergic receptor binding mimics prepared by molecular imprinting. *Journal of Molecular Recognition* 9: 691–696.

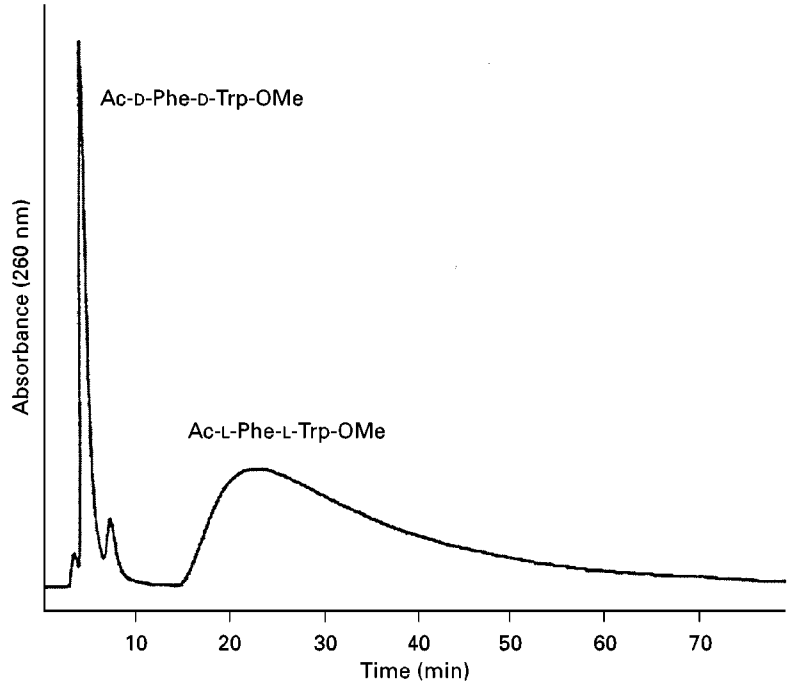


Figure 2 Separation of 10 μg of a mixture of Ac-L-Phe-L-Trp-OMe and Ac-D-Phe-D-Trp-OMe on a *poly*(methacrylic acid-*co*-EDMA) CSP (4.6 × 200 mm column) imprinted with Ac-L-Phe-L-Trp-OMe. Isocratic elution at 1 mL min^{-1} with $CHCl_3$-HOAc (99 : 1). Attentuation was increased 10-fold at 10 min. (Adapted from Ramström O, Nicholls IA and Mosbach K (1994) *Tetrahedron*: *Asymmetry* 5: 649–656, © 1994, with permission from Elsevier Science, UK.)

print molecule by one methylene unit, these polymers were able to separate all of the tested structurally related racemates, even if the separations were not as good as with the print molecule and its optical antipode. This shows that the polymer recognizes both the amino-protecting group and the side chain, but that an exact fit is not necessary for enantioseparation in these cases.

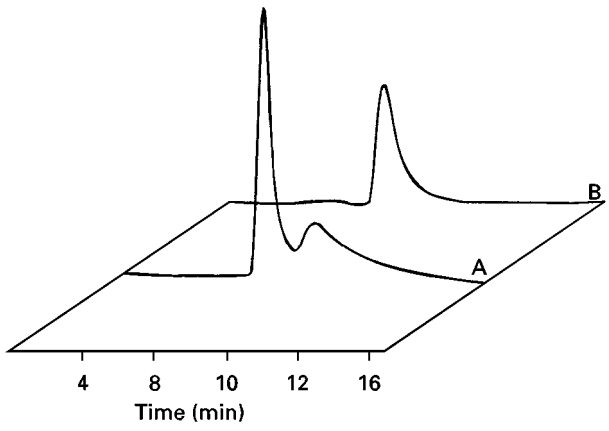
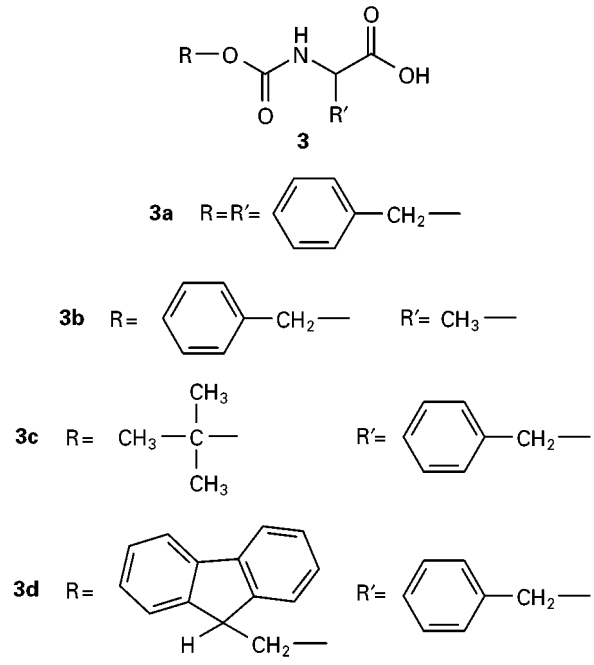


Figure 3 *Poly*(4-vinylpyridine-*co*-EDMA) CSP (4.6 × 200 mm column) imprinted with Z-L-aspartic acid. (A) 20 μg of racemic Z-aspartic acid was applied. Isocratic elution at 0.5 mL min^{-1} with tetrahydrofuran–HOAc (24 : 1) and detection at 260 nm. (B) 20 μg of racemic Z-glumatic acid was applied. Isocratic elution at 0.5 mL min^{-1} with tetrahydrofuran– HOAc (199 : 1) and detection at 260 nm. (Adapted from Kempe M, Fischer L and Mosbach K (1993) *Journal of Molecular Recognition* 6: 25–29, © 1993, with permission from John Wiley & Sons, UK.)



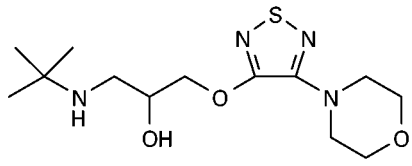
In a study on β-adrenergic blockers, (*S*)-timolol was imprinted in EDMA-based polymers. When the functional monomer was methacrylic acid, the

Table 2 Separation of racemic amino acid derivatives on Z-L-Phe-OH-imprinted CSPs

Racemate	poly *(MAA*-co-*EDMA)*[a,b,c] α	poly *(MAA*-co-*TRIM)*[a,b,d] α
Z-Phe-OH	1.84	2.49
Boc-Phe-OH	1.31	1.78
Fmoc-Phe-OH	1.21	1.66
Z-Ala-OH	1.28	1.59

[a]EDMA, Ethylene glycol dimethacrylate; MAA, methacrylic acid; TRIM, trimethylolpropane trimethacrylate.
[b]The separation factors were calculated as $\alpha = k_L / k_D$; $k = (t - t_0)/t_0$; t is the retention time of the analyte and t_0 is the retention time of unretained compound (the void).
[c]Data from Kempe M and Mosbach K (1994) Chiral recognition of N^a-protected amino acids and derivatives in non-covalently molecularly imprinted polymers. *International Journal of Peptide and Protein Research* 44: 603–606.
[d]Data from Kempe M (1996) Antibody-mimicking polymers as chiral stationary phases in HPLC. *Analytical Chemistry* 68: 1948–1953.

polymer was able to resolve not only racemic timolol (**4**), but also racemic propanolol (**5**). In contrast to this, a timolol-imprinted polymer prepared with itaconic acid as the functional monomer instead of methacrylic acid could only resolve racemic timolol out of a number of racemates of structurally related β-blockers (**Figure 4**). This clearly demonstrates that the selectivity of MIPs can be highly dependent on the functional monomer used.



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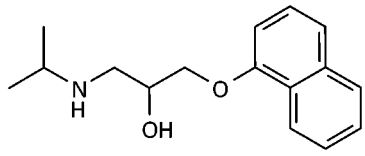

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A comparison of six different CSPs, all imprinted with the same print molecule (Z-L-phenylalanine), confirms that the choice of monomers is important for the selectivity of the resulting polymers (**Table 3**). The selectivity of EDMA-based polymers was higher when vinylpyridines were used, either alone or together with methacrylic acid, than when methacrylic acid was used alone. Methacrylic acid interacts with the print molecule through hydrogen bonds. The beneficial effect of vinylpyridine is attributed to strong ionic interactions between the carboxy groups of the print molecule and the pyridinyl groups. The polymer prepared with acrylamide also showed a higher selectivity than the one prepared with methacrylic acid. Acrylamide forms strong hydrogen bonds even in a polar solvent such as acetonitrile.

It is noteworthy that the load capacity and the resolving capability increased when the trifunctional cross-linker pentaerythritol triacrylate (PETRA) was used instead of EDMA. The same features have been seen with polymers prepared with trimethylolpropane trimethacrylate (TRIM), another trifunctional cross-linker. *Poly*(methacrylic acid-*co*-TRIM) imprinted with a dipeptide was able to resolve 1 mg of the racemate with almost baseline separation (analytical column: 4.6 × 250 mm) (**Figure 5**).

(*S*)-Naproxen (**6**), a nonsteroidal anti-inflammatory drug, has been imprinted in *poly*(4-vinylpyridine-*co*-EDMA) by two different approaches. Bulk polymerization followed by grinding and sieving resulted in highly irregular particles and a two-step swelling and polymerization method gave uniformly sized beads. Both materials, used in the chromatographic mode, were able to separate naproxen from the related ibuprofen (**7**) and ketoprofen (**8**). The polymers were also able to resolve racemic naproxen, but not the racemates of ibuprofen and ketoprofen (**Figure 6**). Even

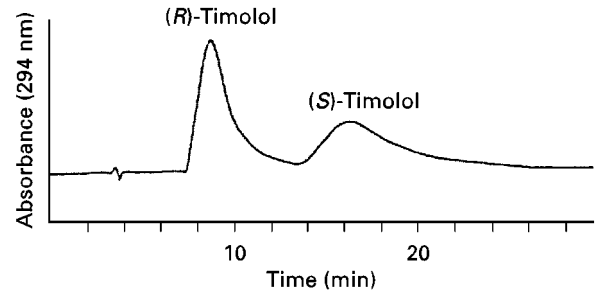

Figure 4 Separation of 20 μg of racemic timolol on a *poly*(itaconic acid-*co*-EDMA) CSP (4.6 × 200 mm column) imprinted with (*S*)-timolol. Isocratic elution at 1 mL min^{-1} with EtOH–tetrahydrofuran–HOAc (5 : 4 : 1). (Adapted from Fischer L, Müller R, Ekberg B and Mosbach K (1991) *Journal of the American Chemical Society* 113: 9358–9360, © 1991, with permission from the American Chemical Society, USA.)

Table 3 Chiral separation of racemic Z-Tyr-OH on molecularly imprinted CSPs

Polymer[a]	*Separated amount (μg)*	α^b	$R_S{}^c$	f/g[d]
poly(AA-*co*-EDMA)[e]	40	3.62	2.52	n.d.
poly(MAA-*co*-EDMA)[f]	10	1.82	n.d.	0.50
poly(4VPy-*co*-EDMA)[g]	20	4.00	1.53	0.94
poly(2VPy-*co*-EDMA)[f]	10	3.81	1.90	0.95
poly(MAA-*co*-2VPy-*co*-EDMA)[f]	10	4.32	1.90	0.97
poly(MAA-*co*-PETRA)[h]	100	2.86	5.47	1.00
poly(MAA-*co*-PETRA)[h]	1000	2.06	n.d.	0.93

[a]AA, Acrylamide; EDMA, ethylene glycol dimethacrylate; MAA, methacrylic acid; 2VPy, 2-vinylpyridine; 4VPy, 4-vinylpyridine; PETRA, pentaerythritol triacrylate.
[b]The separation factors were calculated as $\alpha = k'_L/k'_D$; $k' = (t - t_0)/t_0$; t is the retention time of the analyte and t_0 is the retention time of unretained compound (the void).
[c]The resolution factors (R_S) were calculated according to Wulff G, Poll HG and Minárik M (1986) Enzyme-analogue built polymers. XIX. Racemic resolution on polymers containing chiral cavities. *Journal of Liquid Chromatography* 9: 385–405.
[d]The resolution factors (*f*/*g*) were calculated according to Meyer VR (1987) Some aspects of the preparative separation of enantiomers on chiral stationary phases. *Chromatographia* 24: 639–645.
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[f]Data from Ramström O, Andersson LI and Mosbach K (1993) Recognition sites incorporating both pyridinyl and carboxy functionalities prepared by molecular imprinting. *Journal of Organic Chemistry* 58: 7562–7564.
[g]Data from Kempe M, Fischer L and Mosbach K (1993) Chiral separation using molecularly imprinted heteroaromatic polymers. *Journal of Molecular Recognition* 6: 25–29.
[h]Data from Kempe M (1996) Antibody-mimicking polymers as chiral stationary phases in HPLC. *Analytical Chemistry* 68: 1948–1953.

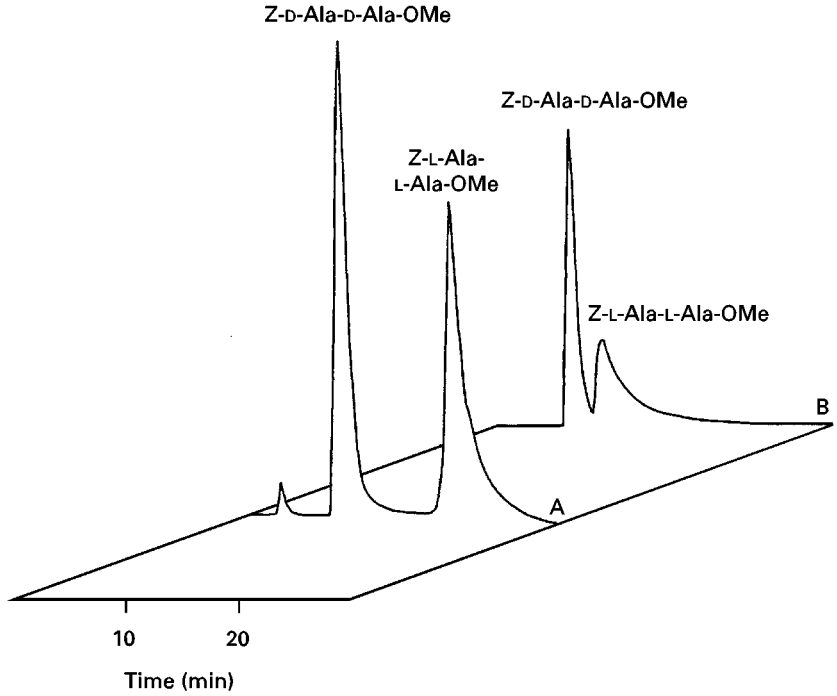


Figure 5 Separation of mixtures of Z-L-Ala-L-Ala-OMe and Z-D-Ala-D-Ala-OMe on a *poly*(methacrylic acid-*co*-TRIM) CSP (4.6 × 250 mm column) imprinted with Z-L-Ala-L-Ala-OMe. (A) 100 μg was applied. Gradient elution at 1 mL min^{-1} with $CHCl_3$-HOAc (99.75 : 0.25) and $CHCl_3$-HOAc (4 : 1) (= B). Gradient: 0–10 min, 0% B; 10–18 min, 0–5% B; 18–22 min, 5% B; 22–24 min 5–0% B. Detection at 260 nm. (B) 1 mg was applied. Isocratic elution at 1 mL min^{-1} with $CHCl_3$-HOAc (99.75 : 0.25). Detection at 260 nm. (Adapted from Kempe M (1996) *Analytical Chemistry* 68: 1948–1953, © 1996, with permission from the American Chemical Society, USA.)

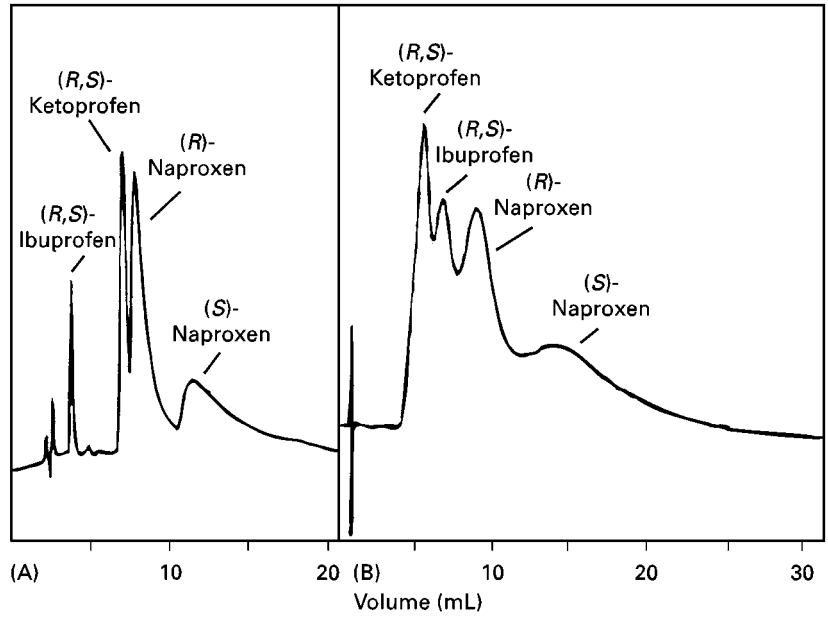
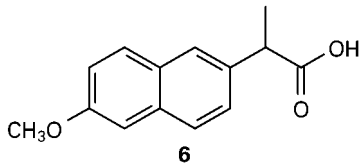


Figure 6 Separation of racemic mixtures of naproxen, ibuprofen and ketoprofen on *poly*(4-vinylpyridine-*co*-EDMA) CSPs (4.6 × 100 mm columns) imprinted with (*S*)-naproxen. (A) The column was packed with particles prepared by grinding and sieving a bulk polymer. Isocratic elution at 0.1 mL min^{-1} with tetrahydrofuran–heptane–HOAc (250 : 250 : 1) and detection at 260 nm. (Adapted from Kempe M and Mosbach K (1994) *Journal of Chromatography A* 664: 276–279, © 1994, with permission from Elsevier Science, UK.) (B) The column was packed with beads prepared by a two–step swelling and polymerization method. Isocratic elution at 1.0 mL min^{-1} with CH_3CN–phosphate buffer (20 mmol L^{-1}, pH 4.0) (1 : 1) and detection at 254 nm. (Adapted from Haginaka J, Takehira H, Hosoya K and Tanaka N (1997) *Chemistry Letters*, 555–556, © 1997, with permission from the Chemical Society of Japan.)

if comparisons of the two chromatograms cannot be done because of differing flow rates, it is not obvious that the chromatographic efficiency was better with the uniformly sized beads (**Figure 6**B) than with the irregular particles (**Figure 6**A). This may be due to impairment on the selectivity by water interfering with the monomer–print molecule complex, since water was used as the suspension medium in the two-step swelling and polymerization method.



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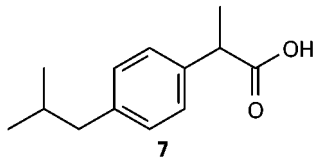

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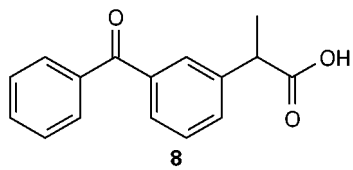

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In general, the polymerizations in noncovalent molecular imprinting have to be done in nonaqueous solutions to prevent water molecules from interfering with the interactions between the monomers and the templates, as previously discussed. Several reports, however, show that the chromatography can be performed efficiently with buffered aqueous eluents.

An approach has been developed which allows both the imprinting and the chiral separation of free amino acids to be carried out in aqueous solutions. The recognition was based on metal coordination–chelation interactions using *N*-(4-vinylbenzyl)iminodiacetic acid as the functional monomer. The method worked best for aromatic amino acids (**Figure 7**).

TLC

MIPs selective for phenylalanine anilide have been evaluated as stationary phases in TLC. Glass backing plates were coated with mixtures of finely ground MIP particles and binders. The plates showed preferential retardation of the enantiomer used as the print molecule (**Figure 8**). The R_F values of both enantiomers on nonimprinted polymers were higher than those observed on the imprinted polymers. The separations suffered from spot broadening, which was attributed to the heterogeneity of the recognition sites.

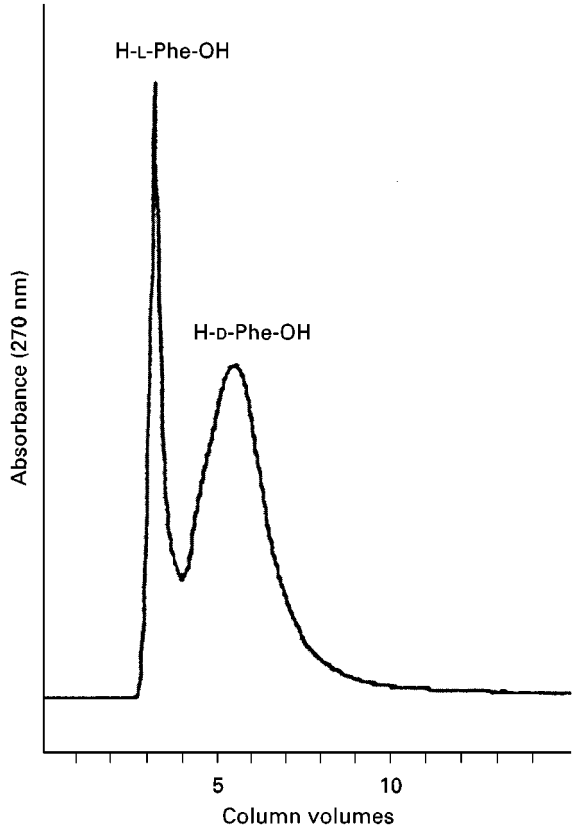

Figure 7 Separation of 17 μg of racemic phenylalanine on *poly*(Cu(II)[*N*-(4-vinylbenzyl)]iminodiacetate-*co*-EDMA) grafted to silica particles (4.6 × 50 mm column). The polymer was imprinted with D-phenylalanine. Isocratic elution at 1 mL min^{-1}, 50°C with 1.5 mmol L^{-1} glycine in water. (Adapted from Vidyasankar S, Ru M and Arnold FH (1997) *Journal of Chromatography A* 775: 51–63, © 1997, with permission from Elsevier Science, UK.)

The technique is attractive because it is simple, fast and allows multiple analyses in the same run.

CE and CEC

Chiral separation by CE and CEC is achieved using a chiral selector which is either free in the mobile phase or immobilized to the stationary phase. MIPs can be used as the selector in both approaches. Molecularly imprinted capillary columns have been prepared by various approaches:

1. packing performed MIP particles into the capillaries
2. dispersion polymerization *in situ* in the capillaries
3. incorporating preformed MIP particles into polyacrylamide gels
4. *in situ* polymerization on the inner walls of the capillaries
5. filling the capillaries with a monolithic polymer with continuous pores by *in situ* polymerization.

Figure 9A shows the separation of racemic propranolol by CEC on a capillary column filled with *poly*(methacrylic acid-*co*-TRIM), polymerized *in situ* using (*R*)-propanolol as the print molecule. Several attractive features make this system look promising for the future: very low consumption of the print molecule (in this case only 10 μg), fast preparation of the capillaries (3 h) and fast separation (less than 2 min). In Figure 9B, MIP particles selective for (*S*)-propanolol were added to the mobile phase in a CE separation.

Future Developments

Molecular imprinting is a technique which has great potential. MIPs have found many applications, and many more are likely to be developed. One of the first applications investigated was CSPs, the subject of this chapter. A number of polymer systems have been developed and these have been used to imprint different classes of compounds.

To be able to attribute the binding of an MIP to an imprinting effect it is of the utmost importance to show that specific recognition sites were formed due to the presence of the print molecules during the polymerization. This is done by comparison with appropriate reference polymers. Polymers prepared without print molecules are not always the best choice, since the physical properties (surface area,

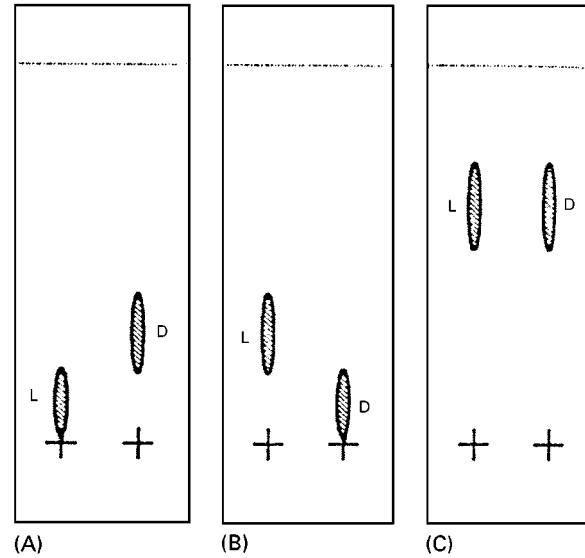

Figure 8 Separation of L- and D-phenylalanine anilide on TLC plates covered with *poly*(methacrylic acid-*co*-EDMA) imprinted with (A) L-phenylalanine anilide; (B) D-phenylalanine anilide and (C) no print molecule. Elution with CH_3CN–HOAc (99 : 5). (Adapted from Kriz D, Berggren Kriz C, Andersson LI and Mosbach K (1994) *Analytical Chemistry* 66: 2636–2639, © 1994, with permission from the American Chemical Society, USA.)

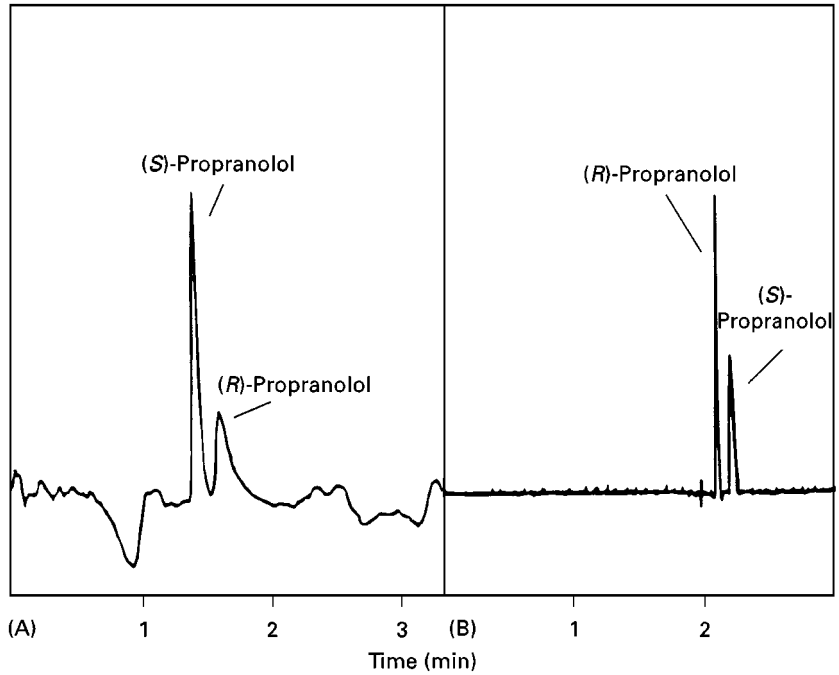

Figure 9 (A) Capillary electrochromatography (CEC). Separation of racemic propranolol on a *poly*(methacrylic acid-*co*-TRIM) CSP (75 μm × 350 mm capillary column) imprinted with (*R*)-propranolol. The sample was injected electrokinetically (5 kV, 3 s) and was separated at a constant voltage of 30 kV. The electrolyte was CH_3CN–acetate buffer (4 mol L^{-1}, pH 3.0) (8 : 2). Detection at 214 nm. The capillary was thermostated to 60°C and an overpressure of 7 bar was applied. (Adapted from Schweitz L, Andersson LI and Nilsson S (1997) *Analytical Chemistry* 69: 1179–1183, © 1997, with permission from the American Chemical Society, USA.) (B) Capillary electrophoresis (CE). Separation of racemic propranolol using 0.05% (w/v) *poly*(*N*-acryloylalanine-*co*-EDMA) particles imprinted with (*S*)-propranolol as a chiral additive in the background electrolyte (100 μm × 470 mm capillary column). The sample was injected by a 3 s pressure injection and was separated at a constatnt voltage of 15 kV. The electrolyte was 5 mmol L^{-1} phosphate buffer, pH 7.0. Detection at 210 nm. Temperature: 25°C. (Adapted from Walshe M, Garcia E, Howarth J, Smyth MR and Kelly MT (1997) *Analytical Communications* 34: 119–122, © 1997, with permission from the Royal Society of Chemistry, UK.)

porosity, etc.) of these polymers are often different from those of imprinted polymers. Reference polymers prepared with the optical antipode or a racemic mixture as the print species are preferred. The selectivity will be reversed when using the optical antipode, and a racemic mixture will give a polymer incapable of separating the two enantiomers (unless the monomers are chiral).

The goal of an endeavour involving chromatographic separation is to achieve the best possible performance with respect to selectivity, resolution, load capacity and analysis time. Much research effort on MIPs has therefore focused on improving the chromatographic performance. The use of monodisperse spherical beads instead of irregular particles improves the efficiency and investigations in this direction with MIPs have already given promising results. Another issue that needs to be investigated further is the heterogeneity of the binding sites. A more homogeneous population of sites would improve the chromatographic performance. The load capacity of MIPs has been shown to be improved by the use of trifunctional cross-linkers such as TRIM and PETRA instead of the bifunctional EDMA. These findings look promising for future developments of MIPs for semipreparative and preparative purifications.

Molecular imprinting is an expanding area attracting an increasing number of scientists, in both industry and academia. This is evidenced by the fact that three-quarters of all papers on molecular imprinting were published during the last decade and one-third during 1996–1997. The rapid development of this technique is likely to result in many breakthroughs within the next few years.

See also: **II / Affinity Separation:** Imprint Polymers.

Further Reading

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Protein Stationary Phases

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In the early 1950s, it was reported that the binding of the enantiomers of an anionic azo-dye to bovine serum albumin (BSA) or human serum albumin (HSA) was different. Further, it was reported that L-tryptophan was bound to serum albumin (bovine mercaptoalbumin) more strongly than the D-form. These studies clearly indicated the possibility of enantioselective binding of ligands to proteins. In 1973, BSA-Sepharose was used for the separation of tryptophan enantiomers; this is the first report on the use of a protein stationary phase for chiral resolution purposes. With this stationary phase, D- and L-tryptophan were clearly resolved, and the D-form was eluted first, as shown in the previous binding studies in solution. In the following years, high performance liquid chromatography (HPLC) chiral stationary phases based on a protein were developed and used to separate a variety of enantiomers. HPLC chiral stationary phases based on a protein are of special interest because of their unique properties of stereoselectivity and because they are suited for separating a wide range of enantiomeric mixtures. Protein-based stationary phases developed so far have included albumins such as BSA and HSA, enzymes such as trypsin, α-chymotrypsin, lysozyme and pepsin, and glycoproteins such as α_1-acid glycoprotein (AGP) from human or bovine serum, cellobiohydrolase I (CBH I), ovomucoid (in fact, ovoglycoprotein), avidin, ovotransferrin and flavoprotein (riboflavin-binding protein). The physical properties of these proteins are shown in **Table 1**. Chiral stationary phases based on BSA, HSA, pepsin, AGP, CBH I, ovomucoid and avidin are now commercially available. Among those, AGP and ovomucoid-based stationary phases can separate a wide range of weakly acidic, weakly basic and neutral racemates.

The advantages of protein-based stationary phases generally include the use of an aqueous mobile phase, enantioselectivity for a wide range of compounds and direct analysis without derivatization. The disadvantages included low capacity, lack of ruggedness and limited understanding of the chiral recognition mechanism. Thus, the protein-based stationary phases are useful for analytical purposes, but are not generally applicable to preparative isolation. To stabilize the protein-based stationary phases, chemical modification of the side chains of the amino acids in the protein has been tried. Further, chiral stationary phases based on a protein fragment or protein domain have been prepared. These can be of higher capacity because only the active protein mass is used. Also, it is possible to understand the chiral recognition sites of protein-based stationary phases by investigating whether or not independent chiral binding sites exist on each fragment or domain.

This article deals with the preparation of HPLC chiral stationary phases based on a protein, their chiral recognition properties and the chiral recognition mechanisms of these stationary phases.

Preparation of HPLC Chiral Stationary Phases Based on a Protein

Generally, a protein is bound to derivatized silica gels. The disadvantage of silica-based stationary