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Review

Imprinted chiral stationary phases in high-performance liquid chromatography

Börje Sellergren*

Department of Inorganic Chemistry and Analytical Chemistry, Johannes Gutenberg University, Mainz, Duesbergweg 10–14, 55099 Mainz, Germany

Abstract

Polymers imprinted with chiral templates offer a new generation of tailor-made chiral stationary phases (CSPs) with predictable selectivities. This review summarizes the present state of the art of molecular imprinting to generate tailor-made CSPs and provides an overview of the main factors involved in the manufacturing process that are crucial to the chromatographic performance of the phases.

Keywords: Reviews; Chiral stationary phases, LC; Molecular imprinting; Imprinted chiral stationary phases

Contents

*Tel.: +49-6131-3925-722; fax: +49-6131-3922-710.
E-mail address: borje@ak-unger.chemie.uni-mainz.de (B. Sellergren).

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1. Introduction

The search for more potent drugs with high target specificities, few side effects and high safety has led to a large number of structurally complex drugs exhibiting chirality [1]. Requirements to administer these drugs in enantiomerically pure form has led to an upsurge of technologies aimed at producing the chiral entities on a preparative scale. Techniques for resolution of racemates are dominating and among these, direct resolution by liquid chromatography (LC), relying on enantioselective recognition by a chiral stationary phase (CSP) or a chiral mobile phase additive, is now a standard technique for the generation of pure enantiomers in pharmaceutical R&D (see other chapters in this issue) [2]. During this development techniques are required allowing rapid determination of the optical purity of the product or intermediates. Also for this purpose high-performance liquid chromatography (HPLC), together with gas chromatography (GC) and capillary electrophoresis (CE), is the method of choice.

One direct and straightforward approach to the development of new CSPs is the use of natural (polysaccharides, proteins) or synthetic polymers (polyacrylates with pendant chiral groups, polyamides or helical polymers of one screw sense) [3]. These are readily available and can be used to separate a broad range of racemates. Due to the high site density of the polysaccharide-based phases (modified amylose or cellulose) these are the most common phases used for preparative scale separations. A problem with these as well as other common CSPs is the limited predictability of elution orders and separability, making screening of stationary phase libraries a necessary step in the method development. Polymers imprinted with chiral templates here promises to alleviate these problems offering a new generation of tailor-made CSPs with predictable selectivities [4]. This review will summarize the present state of the art of molecular imprinting to generate tailor-made CSPs and give the reader an overview of the main factors involved in the manufacturing process.

2. Noncovalent molecular imprinting

Molecularly imprinted polymers (MIPs) can be prepared according to a number of approaches that are different in the way the template is linked to the functional monomer and subsequently to the polymeric binding sites (Fig. 1). Thus the template can be linked and subsequently recognized by virtually any combination of cleavable covalent bonds, metal ion coordination or noncovalent bonds. The first example of molecular imprinting of organic network polymers introduced by Wulff was based on a covalent attachment strategy, i.e., covalent monomer-template, covalent polymer-template [5].

Currently the most widely applied technique to generate molecularly imprinted binding sites is represented by the noncovalent route developed by the group of Mosbach [6]. This makes use of noncovalent self-assembly of the template with functional monomers prior to polymerization, free radical polymerization with a crosslinking monomer and then template extraction followed by rebinding by noncovalent interactions. Although the preparation of a MIP by this method is technically simple it relies on the success of stabilisation of the relatively weak interactions between the template and the functional monomers. Stable monomer-template assemblies will in turn lead to a larger concentration of high affinity binding sites in the resulting polymer. The materials can be synthesized in any standard equipped laboratory in a relatively short time and some of the MIPs exhibit binding affinities and selectivities in the order of those exhibited by antibodies towards their antigens. Nevertheless, in order to develop a protocol for the recognition of any given target, all of the alternative linkage strategies have to be taken into account.

Most MIPs are synthesized by free radical polymerization of functional monounsaturated (vinylic, acrylic, methacrylic) monomers and an excess of crosslinking di- or triunsaturated (vinylic, acrylic, methacrylic) monomers resulting in porous organic network materials. These polymerizations have the advantage of being relatively robust allowing polymers to be prepared in high yield using different solvents (aqueous or organic) and at different temperatures [7]. This is necessary in view of the varying solubilities of the template molecules.

The most successful noncovalent imprinting systems are based on commodity acrylic or methacrylic monomers, such as methacrylic acid (MAA), crosslinked with ethyleneglycol dimethacrylate (EDMA).

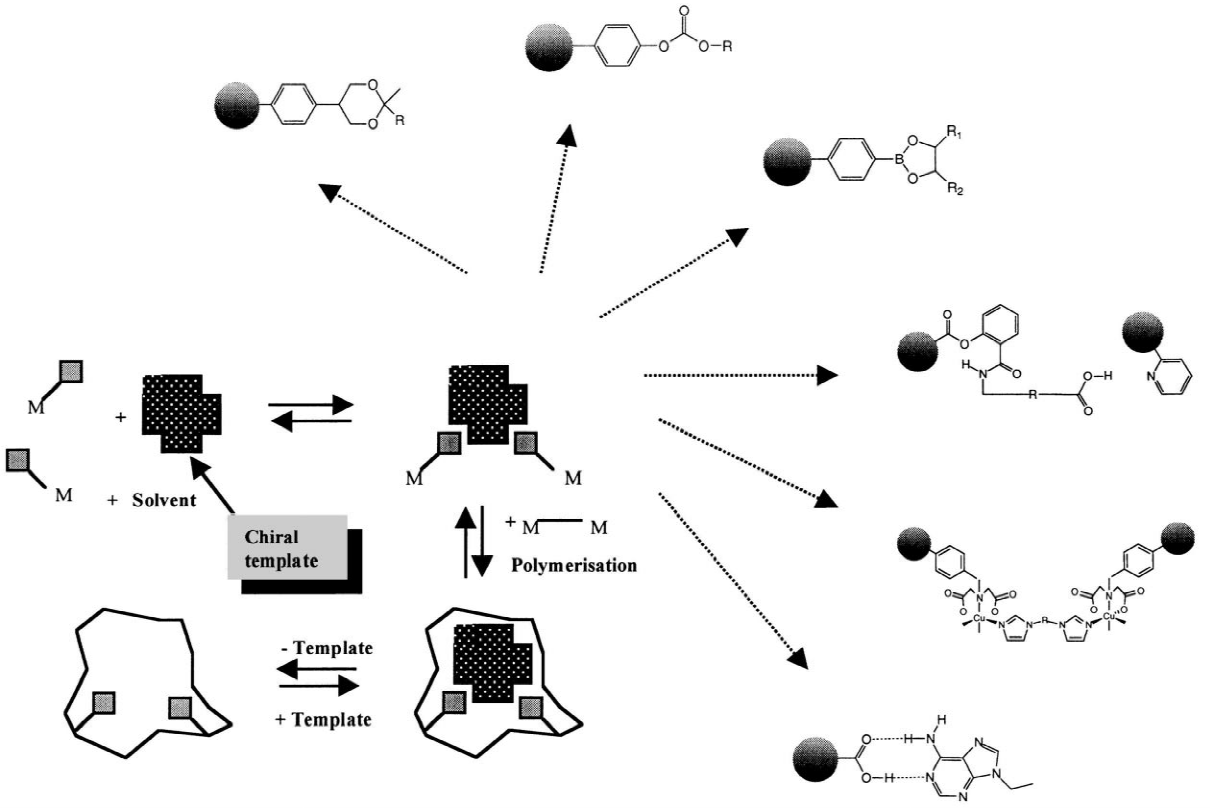


Fig. 1. Approaches to generate imprinted binding sites.

Initially, derivatives of amino acid enantiomers were used as templates for the preparation of imprinted stationary phases for chiral separations (MICSPs) but this system has proven generally applicable to the imprinting of templates allowing hydrogen bonding or electrostatic interactions to develop with MAA [8,9]. The procedure applied to the imprinting with L-phenylalanine anilide (L-PA) is outlined in Fig. 2 [8,9]. In the first step, the template (L-PA), the functional monomer (MAA) and the crosslinking monomer (EDMA) are dissolved in a poorly hydrogen bonding solvent (porogen) of low to medium polarity. The free radical polymerization is then initiated with an azo initiator, commonly azo-*N*,*N*′-bis-isobutyronitrile (AIBN) either by photochemical homolysis below room temperature [9,10] or thermochemically at 60°C or higher [8]. Lower thermochemical initiation temperatures down to 40°C or 30°C is also possible using less stable azoinitiators [10,11]. In the final step, the resultant polymer is crushed by mortar and pestle or in a ball mill, extracted using a Soxhlet apparatus, and sieved to a particle size suitable for chromatographic (25–38 μm) or batch (150–250 μm) applications [9]. The polymers are then evaluated as stationary phases in chromatography by comparing the retention time or capacity factor (k') [12] of the template with that of structurally related analogs (Fig. 3). We will refer to the system shown in Fig. 2 as the L-PA model system.

In the elucidation of retention mechanisms, an advantage of using enantiomers as templates is that nonspecific binding, which affects both enantiomers equally, cancels out. Therefore the separation factor (α) uniquely reflects the contribution to binding from the enantioselectively imprinted sites. As an additional comparison the retention on the imprinted phase is compared with the retention on a nonimprinted reference phase. The efficiency of the separations is routinely characterized by estimating a number of theoretical plates (N), a resolution factor (R_s) and a peak asymmetry factor (A_s) [12]. These

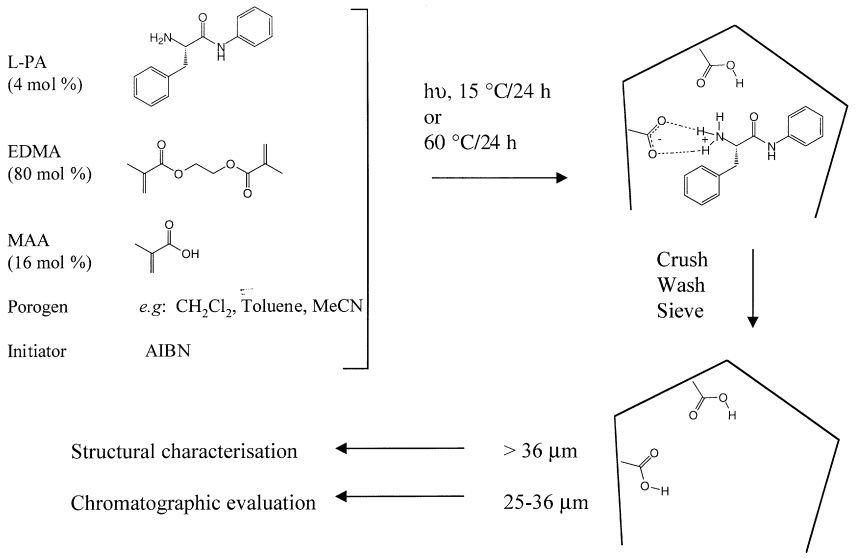


Fig. 2. Preparation of MIPs using L-phenylalanine anilide (L-PA) as template. The L-PA model system.

quantities are affected by the quality of the packing and mass transfer limitations as well as by the amount and distribution of the binding sites.

Some restrictions of this molecular imprinting technique are obvious. The template must be available in preparative amounts, it must be soluble in the monomer mixture and it must be stable and unreactive under the conditions of the polymerization. The solvent must be chosen considering the stability of the monomer-template assemblies and whether it results in the porous structure necessary for a rapid kinetics in the interaction of the template with the

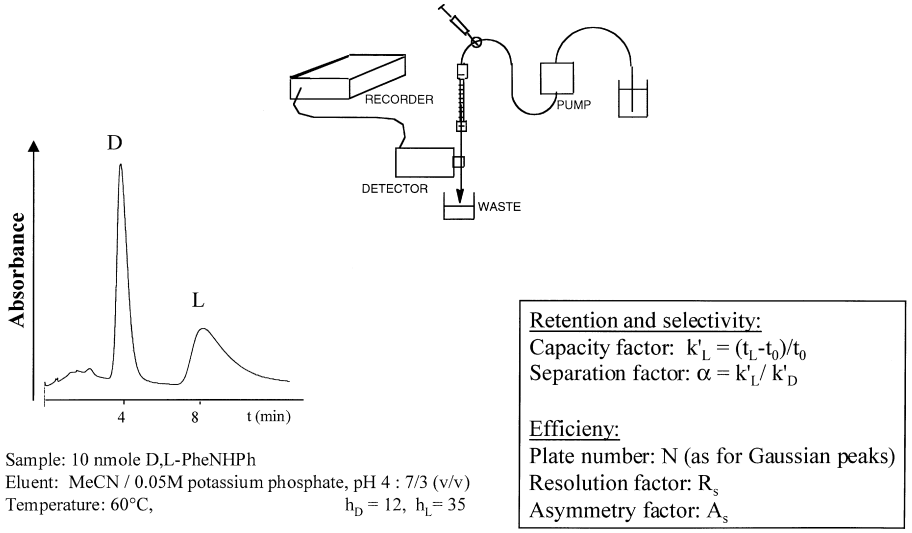


Fig. 3. Principle of the chromatographic evaluation of the recognition properties of MIPs.

Table 1
Examples of racemates successfully resolved on MIPs

Racemate[a]	Separation factor[b]	Resolution factor[b] (R_s)	Note[c]	Ref.
Amino acids				
Phenylalanine	1.6	1.5	[d,e]	[89]
Phenylglycine		0.98		
Tyrosine		0.75		
Amino acid derivatives				
Phenylalanine ethyl ester	1.3	n.d.		[8]
Phenylalanine anilide (PA)	4.9	1.2		[90]
Phenylalanine ethyl amide	2.0	0.5		[8]
p-Aminophenylalanine ethyl ester	1.8	0.8		[8]
Arginine ethyl ester	1.5	n.d.		[91]
Tryptophan ethyl ester	1.8	0.5		[90]
p-Aminophenylalanine anilide	5.7	0.9		[91]
Phenylalanine-*N*-methylanilide (PMA)	2.0	n.d.		[16]
Leucine-β-naphthylamide	3.8	0.7		[18]
N,*N*-Dimethyl-phenylalanine anilide	3.7	1.4		[18]
Proline anilide	4.5	1.0		[18]
Pyridylmethyl-phenylalanine anilide	8.4	1.1		[18]
Pyridoxyl-phenylalanine anilide	2.7	0.4		[18]
Cbz-Glutamic acid	2.5	2.9		[17]
Cbz-Aspartic acid	2.2	1.7		[17]
Cbz-Phenylalanine	2.3	3.1		[67]
Cbz-Alanine	1.9	–	TRIM	[92]
Cbz-Tyrosine	4.3	1.9	VPy-MAA	[93]
Boc-tryptophan	4.4	1.9	VPy-MAA	[93]
Boc-phenylalanine	2.0	1.5	VPy-MAA	[93]
Dansyl-phenylalanine	3.2	1.6	VPy-MAA	[93]
Boc-proline-*N*-hydroxysuccinimide ester	1.3	0.8		[17]
Acetyl-tryptophan methyl ester	3.9	2.2		[93]
Diethyl-2-amino-3-phenyl-propylphosphonate	2.3	n.d.		[94]
Peptides				
Phenylalanylglycine anilide	5.1	0.5		[18]
Cbz-Ala–Ala-OMe	3.2	4.5	TRIM	[92]
Cbz-Ala–Gly–Phe-OMe	3.6	4.2	TRIM	[92]
N-Ac-Phe–Trp-OMe	3.3	>2		[22]
Cbz-Asp–Phe-OMe	2.5		VPY-MAA	[95]
Commercial drugs				
Propranolol	2.8	1.3		[96]
Timolol	2.9	2.0		[96]
Metoprolol	1.08	1.2	TRIM[d]	[76]
Ephedrine	3.4	1.6		[20]
Naproxen	1.7	0.8	VPY	[97]
Ropivacaine	7.7/5.7 [f]		[d]	[98]
Carboxylic acids				
R-(−)-Mandelic acid	1.5	–	VPy	[93]
R-Phenylsuccinic acid	3.6	2.0	VPy	[93]
2-Phenylpropionic acid	High	High	PYAA/DVB[d]	[77]
Amines				
N-(3,5-Dinitrobenzoyl)-methylbenzylamine	1.9	–	MAA/DPGL	[99]
(*R*)-α-Methylbenzylamine	>1.5	1.0		[100]

[a] Each racemate was applied on a polymer (ca. 0.1 μmol per gram dry polymer) imprinted with one antipode of the racemate. The standard mobile phase, consisting of acetonitrile containing various amounts of acetic acid, was used in most cases. Cbz = Carbobenzyloxy, Boc = *tert*.-butyloxycarbonyl.

[b] α was calculated as the ratio of the capacity factor (k') of the template enantiomer to the capacity factor of the other enantiomer. R_s is the resolution factor.

[c] The polymers were prepared using MAA as functional monomer and EDMA as crosslinking monomer if not otherwise noted. VPY = 2- or 4-vinylpyridine. TRIM = trimethylolpropane trimethacrylate. DPGL = (*R*)-*N*,*O*-dimethacryloylphenylglycinol, PYAA = 3-(4-pyridinyl)acrylic acid.

[d] The polymer was evaluated in capillary electrophoresis.

[e] The polymer was imprinted with L-PA.

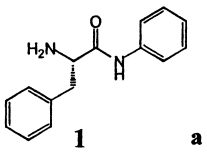
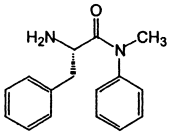
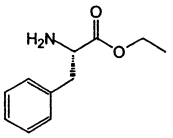
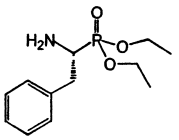
[f] Migration times of the two enantiomers.

binding sites. However if these criteria are satisfied, a robust material capable of selectively rebinding the template can be easily prepared and evaluated in a short time.

3. Structure–binding relationships

A large number of racemates have been successfully resolved on tailor-made MICSPs (Table 1). Using MAA as functional monomer, good recognition is obtained for templates containing Brönsted-basic or hydrogen bonding functional groups close to the stereogenic center. On the other hand, templates containing acidic functional groups are better imprinted using a basic functional monomer such as vinylpyridine. This emphasizes the importance of functional group complementarity when designing the MICSPs. Furthermore, the separation factors are high and higher than those observed for many of the widely used commercial CSPs [3]. However, the columns are tailor-made and the number of racemates resolved equals nearly the number of stationary phases, i.e., each column can resolve only a limited number of racemates. Although the separation factors are high, the resolution factors are low but the performance can often be enhanced by running the separations at higher temperatures [8] and by switching to an aqueous mobile phase (Fig.

Table 2
Examples of highly selective recognition by MIPs[1]

Template	k'_L (1)	α (1)	k'_L (2)	α (2)
 **1** **a**	6.6	4.2	1.05	1.07
 **2** **a**	1.7	1.4	2.1	2.0
 **1** **b**	2.4	2.0	0.9	1.3
 **2** **b**	0.4	1.1	0.8	2.3

[1] The polymers were prepared by the standard procedure using MAA as functional monomer (see Fig. 2) as described elsewhere [16]. (a) Mobile phase: acetonitrile–acetic acid (90:10, v/v). Sample: 0.2 μmol racemate/g. (b) Mobile phase: acetonitrile–water–acetic acid (96.3:1.2:2.5, v/v).

3) [13], or by performing the imprinting in situ in fused-silica capillaries for use in capillary electrochromatography [14]. At low sample loads, the retention on the MICSPs is extremely sensitive to the amount of sample injected indicating overloading of a small amount of high energy binding sites [15]. Moreover the peaks corresponding to the template are usually broad and asymmetric. This is ascribed to the mentioned site heterogeneity together with a slow mass transfer (see the next section).

3.1. High selectivity

MICSPs are often highly selective for their respective template molecule. This is the case for polymer imprinted with L-PA and L-phenylalanine-*N*-methylanilide, respectively (comparing a secondary and tertiary amide as template) (Table 2) [16]. The racemate corresponding to the template was well resolved on the corresponding MICSP whereas the analogue racemate was less retained and only poorly resolved. Similar results were obtained when comparing a polymer imprinted with L-phenylalanine ethyl ester and one with its phosphonate analogue (Table 2) and have also been observed in comparisons of a primary (**1**) and a tertiary (**2**) amine, different in two amino methyl groups, two diacids, *N*-protected aspartic (**3**) and glutamic (**4**) acid, which differed only in one methylene group in the alkyl chain [17,18]. Pronounced discrimination of minor structural differences have also been reported in the imprinting of *N*-protected amino acids as (**5**) and (**6**) [19], aminoalcohols like ephedrine (**7**) and pseudoephedrine (**8**) [20], monosaccharides [21] and peptides like (**9**–**11**) [22]. Since the polymers imprinted with templates containing bulky substituents discriminated against those containing smaller substituents the recognition is not purely size exclusion but instead must be driven by shape complementarity between the site and the substrate, or conformational differences between the derivatives. It was concluded on the basis of ^{1}H nuclear magnetic resonance (NMR) nuclear Overhauser enhancement experiments and molecular mechanics calculations that L-PA and L-Phe-*N*-methylanilide exhibit large conformational differences. Thus the torsional angles between the anilide ring plane and the amide plane, as well as in the *E*–*Z* preference over the amide bond (Fig. 4) are different [16]. The low energy conformer of the anilide has the phenyl group in a *cis* conformation to the carbonyl oxygen with a torsional angle of about 30° whereas in L-Phe-*N*-methylanilide the phenyl group is found in a *trans* conformation twisted almost 90° out of the amide plane. This will result in a different arrangement of the functional groups at the site. In this context it is interesting to note (Table 2) that the polymer imprinted with the *N*-methylanilide is less selective for its template, i.e., a lower separation factor is seen for the template compared to what is observed using the L-PA imprinted polymer and furthermore, a significant separation of the enantiomers of D,L-PA is also observed. This can be explained considering the smaller space requirements of D,L-PA that thus can be forced into a conformation matching the site of the *N*-methylanilide.

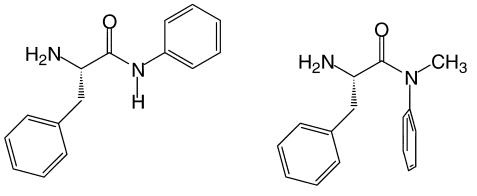
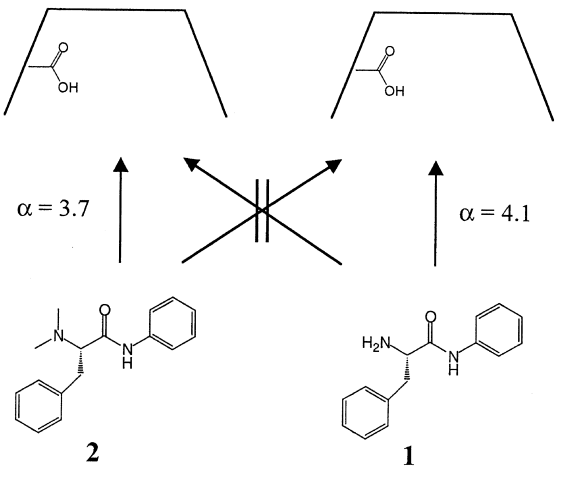
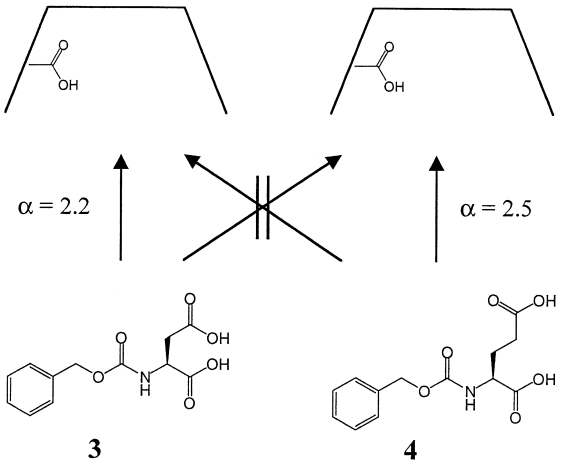
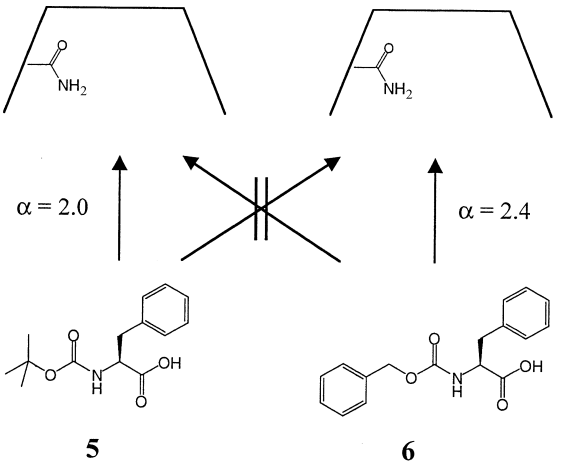
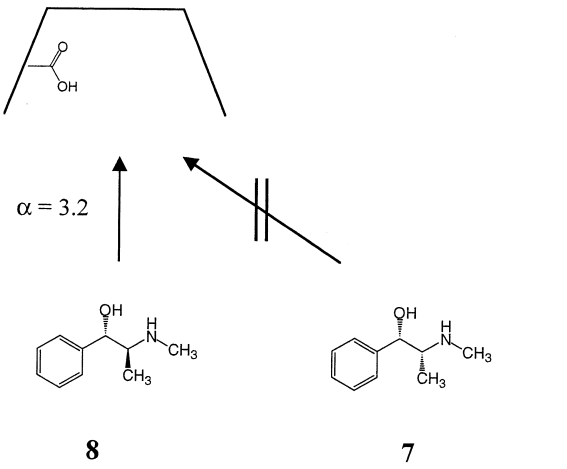


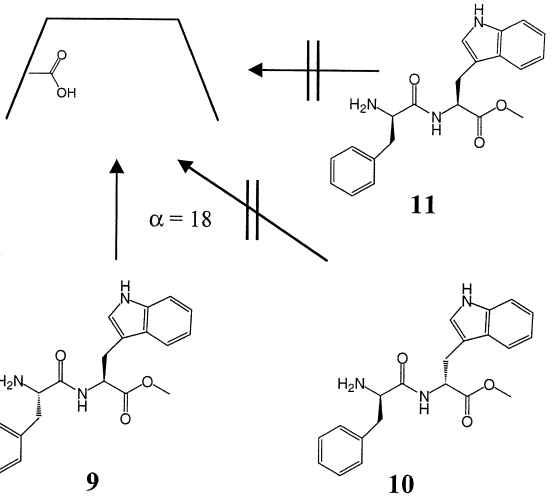
Fig. 4. Minimum energy conformations of L-PA and L-phenylalanine-*N*-methylanilide (L-PMA) based on molecular mechanics calculations and UV and NMR spectroscopic characterizations. From Lepistö and Sellergren [16].











3.2. Low selectivity

Numerous examples of MICSPs that are capable of resolving more than the racemate corresponding to the template have been reported [23,24]. In these cases some structural variations are tolerated without seriously compromising the efficiency of the separation. For instance, a polymer imprinted with L-phenylalanine anilide resolved amino acid derivatives with different side chains or amide substituents [23]. Anilides of all aromatic amino acids were resolved as well as β-naphthylamides and *p*-nitroanilides of leucine and alanine (Table 3). Furthermore, in aqueous mobile phases, the free amino acid phenylalanine could also be baseline resolved on an L-PA imprinted polymer [24]. Apparently, substitution of groups that are not involved in potential binding interactions only leads to a small decrease in

Table 3
Resolution of amino acid derivatives on a MIP imprinted with L-phenylalanine anilide (L-PA)[a]

Racemate	k'_L	α
Phenylalanine anilide	3.5	2.3
Tyrosine anilide	2.9	2.2
Tryptophane anilide	2.4	2.0
Phenylalanine *p*-nitroanilide	3.1	2.1
Leucine *p*-nitroanilide	2.1	1.6
Alanine *p*-nitroanilide	2.0	1.6

[a] Data taken from Ref. [23].

enantioselectivity. Also it was noted that the dipeptide, D,L-phenylalanylglycine anilide was resolved, while glycyl-D,L-phenylalanine anilide was not. This observation emphasizes the importance of the spatial relationship between the functional groups at the sites and indicates that substitutions made at some distance away from the center of chirality are allowed.

4. Adsorption isotherms and site distribution

Adsorption isotherms can yield important information concerning binding energies, modes of binding and site distributions in the interaction of small molecule ligands with receptors [25]. In the case of MIPs, a soluble ligand interacts with binding sites in a solid adsorbent. The adsorption isotherms are then simply plots of equilibrium concentrations of bound ligand (adsorbate) versus concentration of free ligand. The isotherms can be fitted using various models where different assumptions are made. The most simple is the Langmuir type adsorption isotherm (Eq. (1)) where the adsorbent is assumed to contain only one type of sites, where adsorbate–adsorbate interactions are assumed not to occur and where the system is assumed ideal. This isotherm depends on two parameters: the saturation capacity (site density), q_s, and the adsorption energy, b [26,27]:

$$q = \frac{a_1 C}{1 + b_1 C} \quad (1)$$

$$q = \frac{a_1 C}{1 + b_1 C} + \frac{a_2 C}{1 + b_2 C} \quad (2)$$

$$q = aC^{1/n} \ (a \text{ and } n = \text{numerical parameters}) \quad (3)$$

The bi-Langmuir model (Eq. (2)) or tri-Langmuir model, the sum of two or three Langmuir isotherms, correspond to models that assume the adsorbent surface to be heterogenous composed of two or three different site classes and finally the Freundlich isotherm model (Eq. (3)) with no saturation capacity but instead a complete distribution of sites of different binding energies. Depending on the template-functional monomer system, the type of polymer, the conditions for its preparation and the concentration interval covered in the experiment the adsorption isotherms of MIPs have been well fitted with all the isotherm models [28–31].

Thus most MIPs suffer from a heterogenous distribution of binding sites. In noncovalent imprinting, primarily two effects contribute to the binding site heterogeneity. Due to the amorphous nature of the polymer, the binding sites are not identical, somewhat similar to a polyclonal preparation of antibodies. The sites may for instance reside in domains with different crosslinking density and accessibility [32]. Secondly, this effect is reinforced by the incompleteness of the monomer-template association [8]. In most cases the major part of the functional monomer exists in a free or dimerized form, not associated with the template. As a consequence, only a part of the template added to the monomer mixture gives rise to selective binding sites. This contrasts with the situation in covalent imprinting [28,31–33] or stoichiometric noncovalent imprinting [34] where theoretically all of the template split from the polymer should be associated with a templated binding site. The poor yield of binding sites results in a strong dependence of selectivity and binding on sample load at least within the low sample load regime. For determining the adsorption isotherm the equilibrium concentrations of bound and free template has to be reliably measured within a large concentration interval. Since the binding sites are part of a solid this experiment is relatively simple. Thus it can be done in a batch equilibrium rebinding experiment or by frontal analysis.

One powerful technique for the study of the interactions between solutes and stationary phases and for the investigation of the parameters of these interactions is frontal analysis [35]. This method allows accurate determination of adsorption and kinetic data from simple breakthrough experiments and the technique has proven its validity in a number of previous studies. This has also been used for estimating the adsorption energies and saturation capacities in the binding of templates to MIPs but often the data has been modeled only at one temperature and graphically evaluated using a simple Lang-

muir monolayer model which in most cases gives a poor fit of the data [36–38]. Furthermore, the breakthrough curves are interpreted assuming thermodynamic equilibrium which is often an invalid assumption in view of the slow mass transfer in these systems. Instead based on the mass balance equation and by assuming kinetic and isotherm values to best fit isotherms and elution profiles obtained at different temperatures, a more accurate picture of the thermodynamics and mass transfer data can be obtained [35].

The experimental isotherms are shown in Fig. 5 for the two enantiomers of phenylalanine anilide [27]. The lines in the main figure illustrate the best

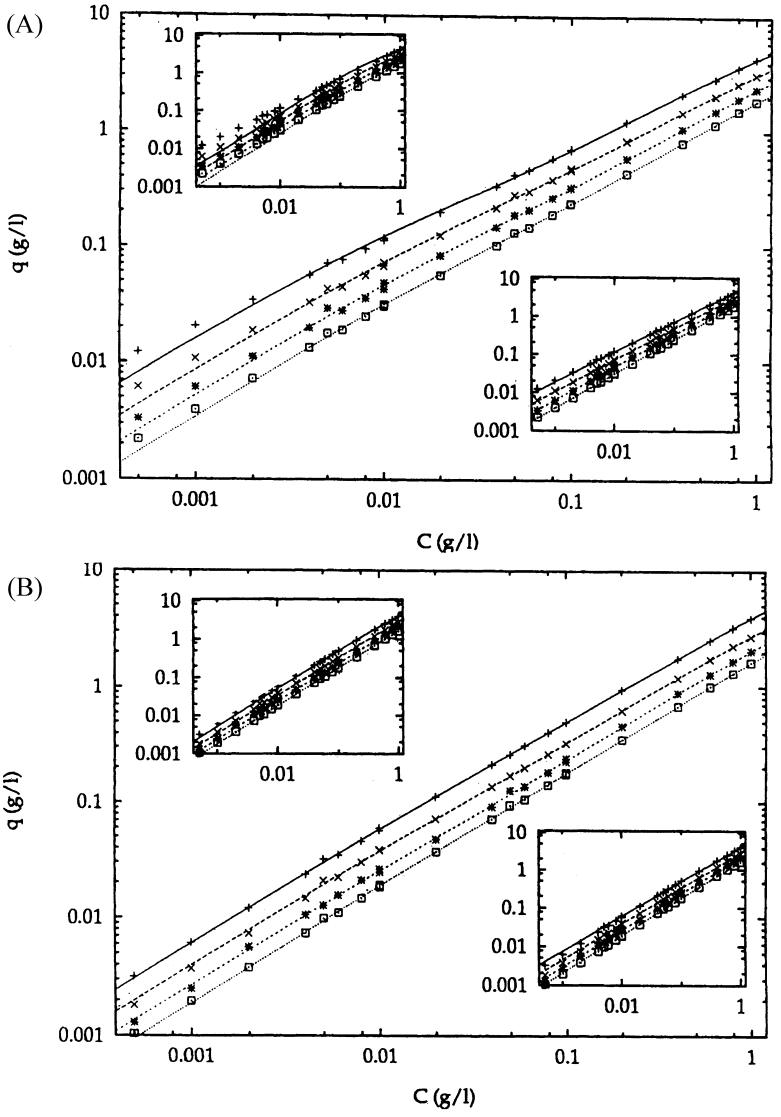


Fig. 5. Frontal analysis of the binding of D- and L-PA to an MIP imprinted with L-PA. The MIP was prepared using methylenechloride as porogen following the standard procedure shown in Fig. 2. Fitting (lines) of the experimental isotherm data (symbols) for L-PA (A) and D-PA (B) to the bi-Langmuir model (main figure), the Langmuir model (left inset) and the Freundlich model (right inset). For the runs at 40°C: solid lines and plus symbols, at 60°C: short dashed lines and stars, at 70°C: dotted lines and squares. Mobile phase: MeCN–0.05 *M* potassium phosphate (KP), pH 5.85 (70:30, v/v). From Sajonz et al. [27].

fits of the experimental data to the bi-Langmuir isotherm equation at 40, 50, 60 and 70°C. The top-left inset shows their best fits to the Langmuir isotherm equation and the bottom-right inset to the Freundlich isotherm equation. The isotherms obtained by fitting the data to the Langmuir equation is of a quality inferior to the other two. The fittings of the data to the Freundlich and to the bi-Langmuir equations are both good. A comparison of the residuals reveals that the different isotherms of D-PA are best fitted to a bi-Langmuir model while the isotherms for L-PA are slightly better fitted to a Freundlich isotherm model, particularly at low temperatures. However, at concentrations higher than 17 μM ($4 \cdot 10^{-3}$ g/l), the isotherm data of L-PA are equally well fitted to the Freundlich and to the bi-Langmuir isotherm models, suggesting the existence of binding sites with higher binding energies ($K > 50\,000\ M^{-1}$). At 40°C for L-PA, the binding constants and site densities are, respectively, 84 M^{-1} and ca. 90 μmol/g for the low affinity sites and 16 000 M^{-1} and 1 μmol/g for the high affinity sites. For D-PA the respective values are 48 M^{-1} and 136 μmol/g for the low affinity sites and 5520 M^{-1} and 0.4 μmol/g for the high affinity sites. These values agree well with values determined in other previous studies [11]. In view of the small saturation capacities observed for D-PA on these sites at the other temperatures studied (50, 60, 70°C) or after thermal annealing of the materials [15], the second site class appears to be specific for L-PA.

For preparative or semipreparative scale enantiomer separations the enantioselectivity and column saturation capacity are the critical factors determining the throughput of pure enantiomer that can be achieved. The above described MICSPs are stable, they can be reproducibly synthesized and exhibit high selectivities, attractive features for such applications. However most MICSPs have only moderate saturation capacities and isocratic elution leads to excessive peak tailing which precludes many preparative applications. Nevertheless, with the L-PA MICSP described above, mobile phases can be chosen leading to acceptable resolution, saturation capacities and relatively short elution times also in the isocratic mode (Fig. 6).

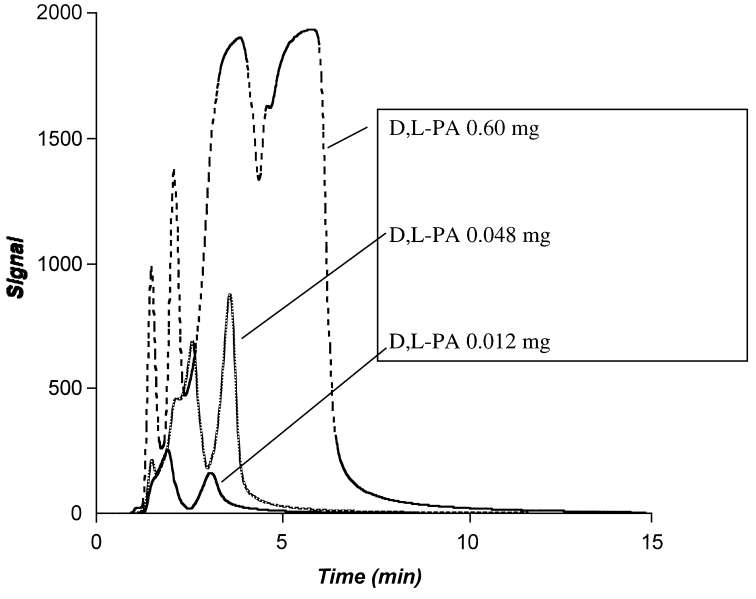


Fig. 6. Overload elution profiles of D,L-PA injected on a column (125×4 mm) packed with the L-PA imprinted stationary phase used in Fig. 5. Mobile phase: MeCN–TFA (0.01%)–water (2.5%). The tendency for fronting and the increase in retention with sample load is attributed in part to saturation of the mobile phase modifier.

5. Adsorption–desorption kinetics and chromatographic band broadening

For most applications of specific molecular recognition elements a fast association dissociation kinetics in the ligand receptor binding is important. In chemical sensors the response time depends on the association rate between the sensor bound receptor and the target analyte whereas the dissociation rate determines if and how fast the sensor can be regenerated [39]. The kinetics thus influences the sample throughput of the analysis, i.e., how many samples that can be analyzed in a certain time interval. Furthermore, in catalysis the binding kinetics will determine the maximum rate of the chemical transformation and in chromatographic separations it will influence the spreading of the chromatographic peaks.

When a solute band passes a chromatographic column it is continuously broadened due to various dispersion processes [40,41]. These include processes that show little or no flow-rate dependence, such as Eddy diffusion or extracolumn effects and flow-rate dependent processes such as axial diffusion, mass transfer processes including mobile phase, intraparticle and stationary phase diffusion and slow kinetic processes upon interaction with the stationary phase. Other factors such as nonlinear binding isotherms and slow desorption kinetics instead affect the shape of the peak [42,43]. Altogether these processes counteract the separation of two compounds and lead to lower resolutions. An understanding of their origin is important in order to improve the separations as well as to gain insight into the kinetics and mechanism of solute retention.

In enantiomer separations of D,L-PA on L-PA imprinted CSPs the dependence of the chromatographic parameters on flow-rate and sample load was studied [44]. Using a thermally annealed stationary phase, a strong dependence of the asymmetry factor (A_s) of the L-form on sample load and a weak dependence on flow-rate suggested that column overloading contributed strongly to the peak asymmetry (Fig. 7). This is to be expected in view of the site heterogeneity discussed in the previous section. However slow kinetic processes is another contributing factor to the pronounced band broadening in the chromatography using MIP-based columns. In view of the high binding constants observed for MIPs, the desorption rate at the high energy binding sites should be much slower than that at the low energy sites. The mass transfer rate coefficients, estimated using a MIP prepared in dichloromethane as diluent, were small and strongly dependent on the temperature and concentration, in particular the rate coefficients corresponding to the imprinted L-enantiomer [27]. Fig. 8 shows overloaded elution profiles obtained for three different injection volumes of solutions of the same concentrations of the two enantiomers (dashed lines).

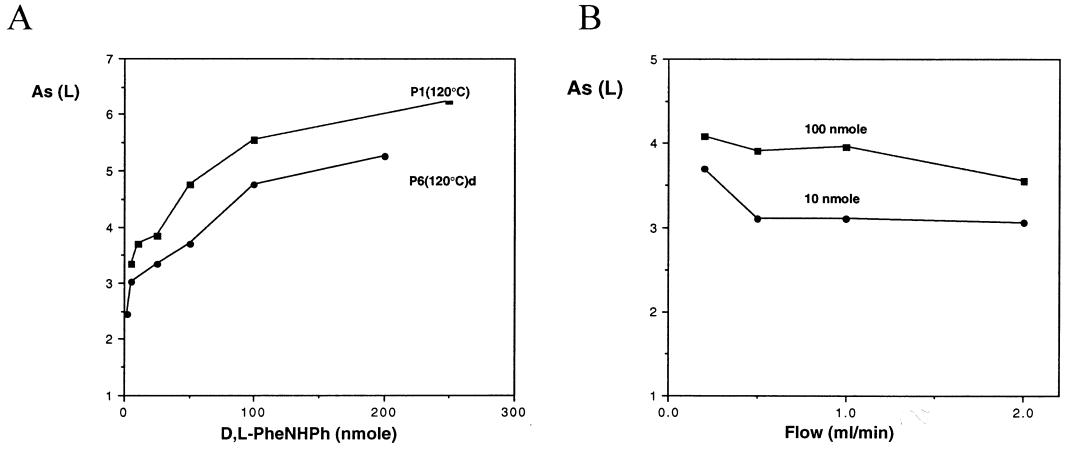


Fig. 7. Asymmetry factor (A_s) of the L-enantiomer versus sample load (A) and versus flow-rate (B) on L-PA imprinted polymers. Flow-rate: 1.0 ml/min. Mobile phase: MeCN–[0.05 *M* KP, pH 7] (7:3, v/v).

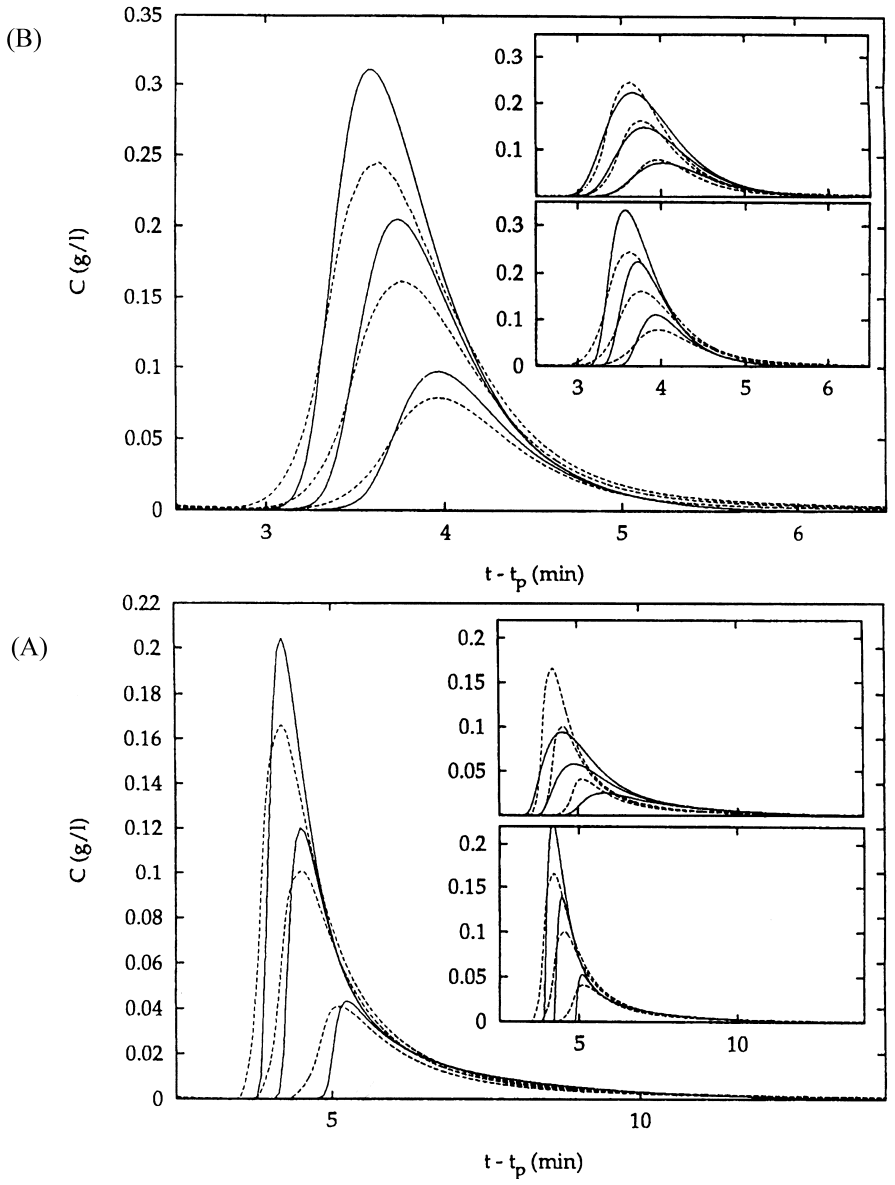


Fig. 8. Example of overloaded elution profiles from the frontal analysis runs in Fig. 5. D,L-PA was injected at sample volumes of 240, 160 and 80 μl at a sample concentration of 1 g/l and a temperature of 40°C. Experimental data: dotted lines. Numerical calculations: solid lines. (A) L-PA, main figure: $k_f = 117.3C^{0.2582}$ min^{-1}, upper inset: $k_f = 10$ min^{-1}, lower inset: $k_f = 110$ min^{-1}. (B) D-PA, main figure: $k_f = 90.9C^{0.1147}$ min^{-1}, upper inset: $k_f = 35$ min^{-1}, lower inset: $k_f = 90$ min^{-1}. Mobile phase: MeCN–0.05 *M* potassium phosphate (KP), pH 5.85 (70:30, v/v). From Sajonz et al. [27].

The solid lines in these figures show the band profiles numerically calculated for a concentration dependent mass transfer coefficient k_f (main figure), a low-concentration value of k_f (upper inset) and a high-concentration value of k_f (lower inset). For the L-enantiomer, the best fit of the data is obtained using the concentration dependent rate constant, while for the D-enantiomer, the best fit is obtained using a constant value of k_f equal to 35 min^{-1} (low concentration value). Recent related studies of the

retention mechanism of both enantiomers of dansyl-phenylalanine on a dansyl-L-phenylalanine MICSP led to similar conclusions [45], although these processes are strongly dependent on the system studied, i.e., template-monomer system, crosslinking monomer, porogen and method of polymerization.

6. Factors to consider in the synthesis of MICSPs

In spite of the fact that molecular imprinting allows materials to be prepared with high affinity and selectivity for a given target molecule a number of limitations of the materials prevent their use in real applications. The main limitations are:

1. Binding site heterogeneity.
2. Extensive nonspecific binding.
3. Slow mass transfer.
4. Bleeding of template.
5. Low sample load capacity.
6. Impractical manufacturing procedure.
7. Poor recognition in aqueous systems.
8. Swelling–shrinkage: may prevent solvent changes.
9. Lack of recognition of a number of important compound classes.
10. Preparative amounts of template required.

It is clear that improvements aiming at increasing the yield of high energy binding sites or modifying the site distribution in other ways will have a large impact on the performance of the materials (affecting limitations 1, 2, 4 and 5). The strategies adopted to achieve this have been focusing either on prepolymerization approaches, aiming at stabilization of the monomer template assemblies prior to polymerization or postpolymerization approaches aiming at modifying the distribution of binding sites by either chemical or physical means. The most important of these factors will now be discussed together with techniques allowing their optimization.

6.1. Factors related to the monomer-template assemblies

It is of obvious importance that the functional monomers strongly interact with the template prior to polymerization, since the solution structure of the resulting assemblies presumably defines the subsequently formed binding sites. By stabilizing the monomer-template assemblies, it is possible to achieve a large number of imprinted sites. At the same time the number of nonspecific binding sites will be minimized, since free functional monomer not associated with the template is likely to be accessible for binding. Considering one particular binding site, the following factors have been identified that are likely to affect the recognition properties of the site (Fig. 9).

The strength and positioning of the monomer-template interactions are of importance for materials with good molecular recognition properties to be obtained. The broad applicability of MAA as a functional monomer is related to the fact that the carboxylic acid group serves well as a hydrogen bond and proton donor as well as a hydrogen bond acceptor [46]. In aprotic solvents such as in acetonitrile, carboxylic acids and amine (bases) form contact hydrogen bonded assemblies where the association strength for a given acid increases with the basicity of the base [47]. Thus templates containing Brönsted-basic or hydrogen-bonding functional groups are potentially suitable templates for the MAA/EDMA system [8,9]. Furthermore, more stable cyclic hydrogen bonds can form with templates containing acid [17], amide [48] or functionalized nitrogen heterocycles [29,30]. The potential for a given monomer template pair to produce templated sites can be predicted by measuring the stability constants, e.g., by spectroscopic techniques, in a homogeneous solution mimicking the monomer mixture prior to polymerization [8,49]. This can ultimately be used as a preliminary screening procedure to search for suitable functional monomers. Thus estimated solution association constants can be correlated with the heterogenous binding constants determined for the polymer (Table 4). For the prepolymerization complexes discussed thus far the electrostatic interactions are sensitive to the presence of polar protic solvents. One exception is the complex formed between carboxylic acids and guanines or amidines [34,50,51]. Here cyclic hydrogen bonded ion-pairs are formed with stability constants that are order of magnitude higher than those previously discussed (Table 4). This allows amidines such as pentamidine (**12**) to be imprinted using isopropanol–

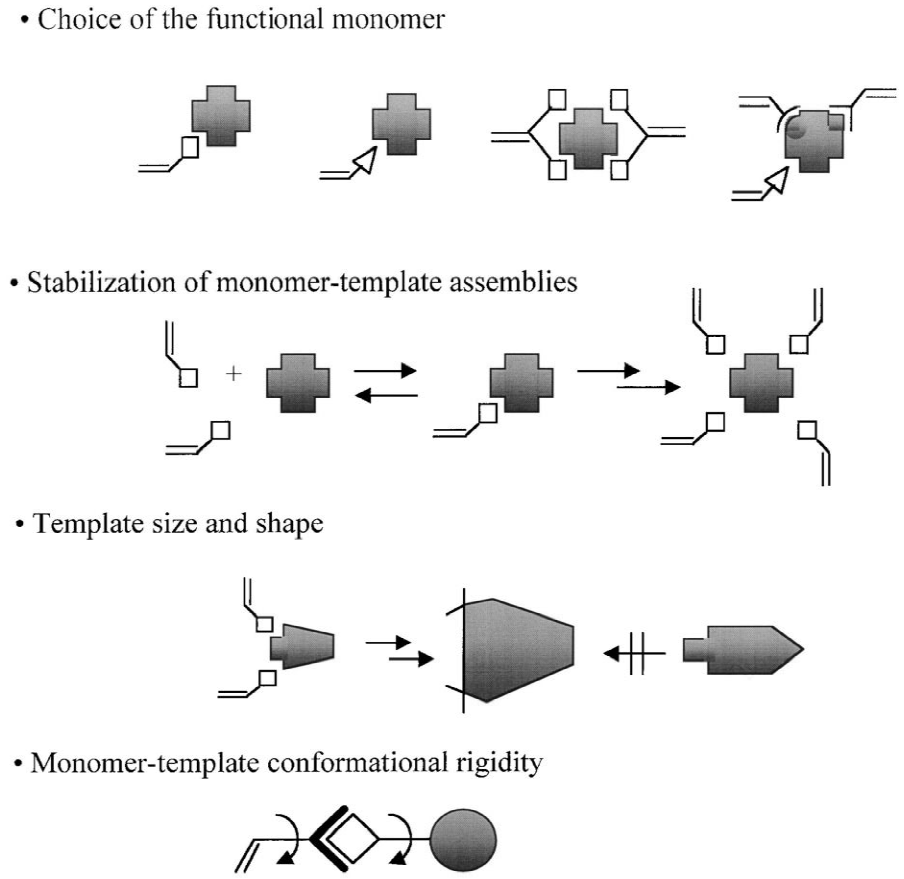


Fig. 9. Factors affecting the recognition properties of MIPs related to the monomer template assemblies.

Table 4
Association constants for complexes between carboxylic acids and nitrogen bases in aprotic solvents and corresponding association constants and site densities for binding of the base to a molecularly imprinted polymer[a]

Acid	Base	Solvent	K_a (M^{-1})	n (μmol/g)	Ref.
Acetic acid	Atrazine	CCl_4	210	–	[101]
Butyric acid	9-Ethyladenine	$CDCl_3$	(1) 114 (2) 41	–	[102]
4-Methylbenzoic acid	(**13**)	$CDCl_3$	$>10^6$	–	[51]
PMAA	Atrazine	$CHCl_3$	(1) $8.3\cdot10^4$ (2) $1.0\cdot10^4$	20 40	[49]
PMAA	9-Ethyladenine	$CHCl_3$	(1) $7.7\cdot10^4$ (2) $2.4\cdot10^3$	20 86	[29]

[a] PMAA refers to polymers imprinted with respective base using MAA as functional monomer.

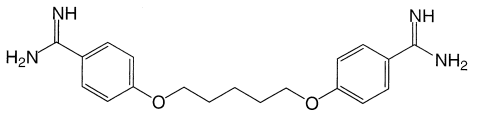
water as a porogenic solvent mixture resulting in polymers that bind pentamidine strongly in aqueous media [52].

Apart from the successful imprinting discussed above the recognition for many templates is far from that is required for the particular application even after careful optimization of the other factors affecting the molecular recognition properties. Often a large excess of MAA in the synthesis step is required for recognition to be observed and then only in solvents of low to medium polarity and hydrogen bond capacity [53]. In fact, in these cases the optimum rebinding solvent is often the solvent used as porogen [54]. Thus the polymer exhibits memory for the template as well as the porogen. Moreover, the excess of functional monomer results in a portion of the functional monomer not being associated with imprinted sites. These sites interact nonselectively with solutes binding to carboxylic acids and limit the degree of separation that can be achieved. Hence MAA is not a universal monomer. Instead, for the recognition of any given target molecule access to functional monomers targeted towards structural features specific for particular compounds or classes of compounds are required.

Based on the structural features of the templates that generate good sites an interesting possibility would be to incorporate these structures in new functional monomers for the recognition of carboxylic acids. This concept is somewhat similar to the reciprocity concept in the design of chiral stationary phases (see other chapters of this issue). Thus Wulff et al. synthesized *N*,*N*′-substituted *p*-vinylbenzamidines (**13**) and showed that these monomers could be used to generate high fidelity sites for the molecular recognition of chiral carboxylic acids [51]. The binding is here strong enough to provide efficient recognition also in aqueous media. Furthermore, due to strong binding the functional monomer is quantitatively associated with the template minimizing the nonspecific binding. Functional group complementarity is thus the basis for the choice of functional monomer. The search for the optimal structural motif to complement the template functionality is preferentially guided by results from the area of host–guest chemistry and ligand–receptor chemistry. Thus cyclodextrins have been used to template binding sites for cholesterol [55] or to enhance the selectivity in the imprinting of enantiomers of amino acids [56]. Based on metal ion coordination of amino acids and *N*-(4-vinylbenzyl)iminodiacetic acid (**14**), imprinting and subsequent chiral separation of free amino acids in aqueous solutions has also been possible [57].

Based on chiral functional monomers such as (**15**), MICSPs can be prepared using a racemic template. Thus using racemic *N*-(3,5-dinitrobenzoyl)-α-methylbenzylamine (**16**) as template, a polymer capable of racemic resolution of the template was obtained [58]. Another chiral monomer based on L-valine (**17**), was used to prepare MIPs for the separation of dipeptide diastereomers [59]. In these cases the configurational chirality inherent in the pendant groups of the polymer are to some extent themselves chiral selectors and the effect of imprinting is merely to enhance the selectivity. A good example of this was shown in the imprinting of *N*-benzyl-L-valine as a bidentate ligand to a styrene-based chiral cobalt complex (**18**) [60]. The strong enantioselectivity of the imprinted polymer should here be viewed with respect to the enantioselectivity of the control polymer.

Alternative approaches to imprint peptides via strong monomer template association have recently been reported although no results of the chromatographic application of these phases have been shown. Strong complexation inducing a β-sheet conformation was possible using a designed functional monomer (**19**) [61]. Peptides can also be imprinted via a sacrificial spacer approach which potentially will result in a high yield of templated sites exhibiting pronounced selectivity towards the target peptide (**20**) [62].



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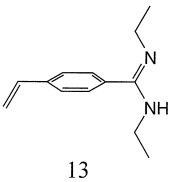

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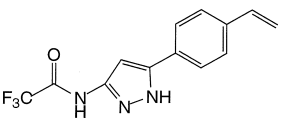

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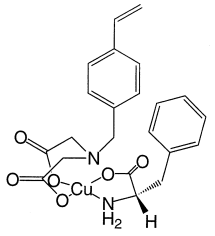

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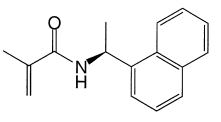

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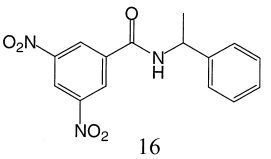

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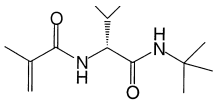

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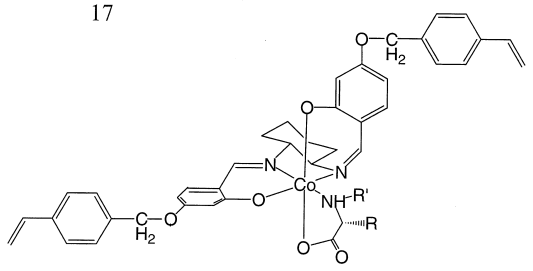

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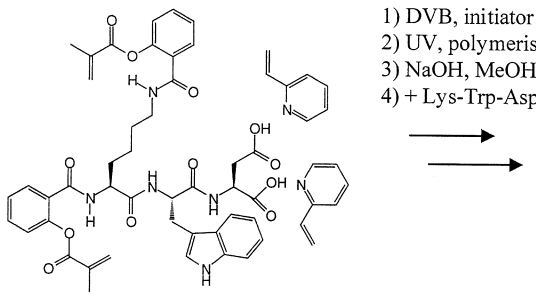

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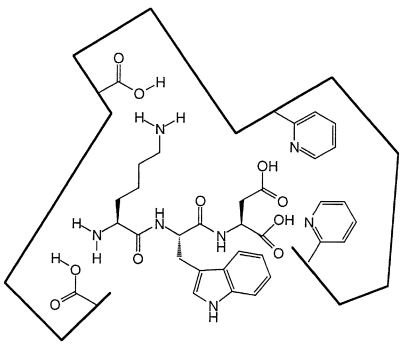
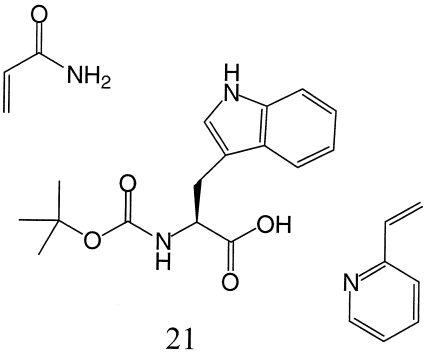


Considering functional group complementarity other commodity monomers may also be used. Thus for templates containing acid groups, basic functional monomers are preferably chosen. The 2- or 4-vinylpyridine (VPY) are particularly well-suited for the imprinting of carboxylic acid templates and provide selectivities of the same order as those obtained using MAA for basic templates [63,64]. These polymers are however susceptible to oxidative degradation and require special handling.

In the imprinting of carboxylic acids and amides high selectivities are also seen using acrylamide (AAM) as a functional monomer [19]. Furthermore, combinations of two or more functional monomers, giving terpolymers or higher polymers, have in a number of cases resulted in better recognition ability than the recognition observed from the corresponding copolymers [58,63–65]. These systems are particularly complex when the monomers contain a donor–acceptor pair, since monomer–monomer association will strongly compete with template-monomer association if neither of the monomers have a particular preference for the template. In a recent paper by Zihui et al., careful optimization showed that a combination of acrylamide and 2-vinylpyridine gave significantly higher enantioselectivities in the imprinting of *N*-protected amino acids than the combination of 2-vinylpyridine with MAA (**21**) [66]. Furthermore, better results were obtained using acetonitrile as the porogen in contrast to other systems where solvents of lower polarity (e.g., toluene, CH_2Cl_2, $CHCl_3$) give the best results. These results show that adequate performance can only be achieved after careful optimization where the related factors are systematically varied.



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6.2. Factors related to polymer structure and morphology

For the formation of defined recognition sites, the structural integrity of the monomer-template assemblies has to be preserved during polymerization to allow the functional groups to be confined in space in a stable arrangement complementary to the template. This is achieved by the use of a high level of crosslinking, usually >80% [11]. The role of the polymer matrix, however, is not only to contain the binding sites in a stable form but also in an accessible form. Porosity is achieved by carrying out the polymerization in presence of a porogen. Most of the crosslinked network polymers used for molecular imprinting have a wide distribution of pore sizes associated with various degrees of diffusional mass transfer limitations and a different degree of swelling. Based on the above criteria, i.e., site accessibility, integrity and stability, the sites can be classified according to different types. The sites associated with meso- and macropores (>20 Å) are expected to be easily accessible compared to sites located in the smaller micropores (<20 Å) where the diffusion is slow. The number of the latter may be higher since the surface area, for a given pore volume, of micropores are higher than that of macropores. One undesirable effect of adding an excess of template is the loss of site integrity due to coalescence of the binding sites, which is related to the extent of template self association. The optimum amount of template is usually about 5% of the total amount of monomer but can be higher when trivinyl monomers such as TRIM (**22**) are used as crosslinkers, where a larger fraction of functional monomer is used [67]. In this case higher sample load capacities have been observed. The amount of template is of course also limited by the solubility and availability of the template although recycling is possible.

Often the materials swell to different extents depending on the type of diluent. The swelling is here normally high in solvents and low in nonsolvents for the polymer. Unfortunately this may lead to large changes in the accessibility and density of the binding sites when the solvent is changed [9].

7. Methods for combinatorial synthesis and screening of large number of MIPs

For a complete optimization of all factors the above described procedure is not practical. In order

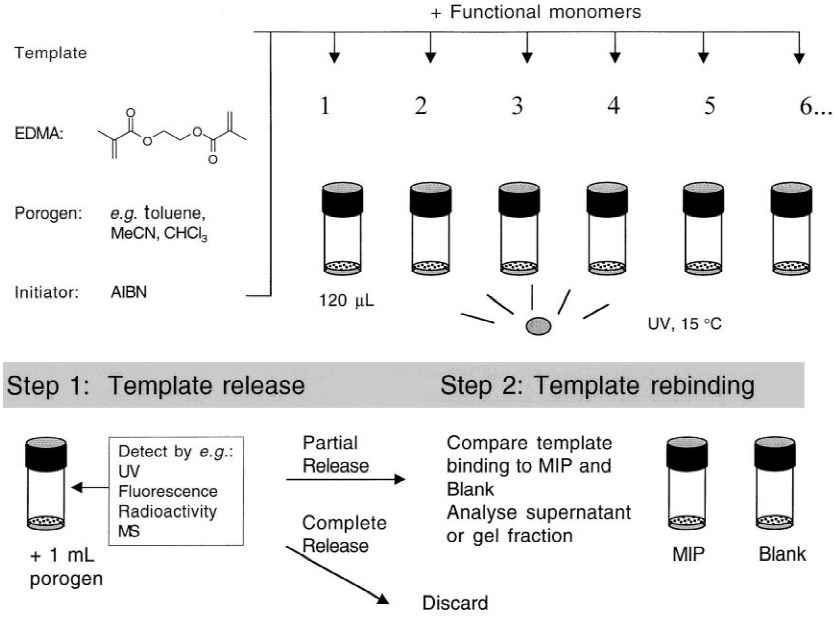


Fig. 10. Combinatorial imprinting technique suitable for automation.

to perform this rapidly parallel synthesis and screening techniques must be developed. These can consist in a scaled down version of the MIPs in vials that can be automatically handled and analyzed in situ (Fig. 10) [103,68].

The principle was demonstrated using triazine herbicides as templates and by varying the type of functional monomer and the monomer composition. With a final batch size of ca. 40 mg of monomer the consumption of monomers and template is significantly reduced and the synthesis and evaluation can take place in standard HPLC autosampler vials. After synthesis the primary assessment is based on quantitative HPLC or UV-absorbance analysis of the amount of template released from the polymer in the porogenic solvent. Thus in the case of a rapid and quantitative release the resulting polymer cannot be expected to rebind a significant amount of the template and may thus be discarded. After having established useful functional monomers a secondary screening for selectivity is performed. Here the rebinding of the template to the MIPs was investigated in parallel to the rebinding to a corresponding control nonimprinted MIP [68]. Alternatively an internal standard, structurally related to the template, may be added and the differential binding investigated [103]. An important question is whether the equilibrium rebinding results reflect the selectivity observed when investigating an upscaled batch in the chromatographic mode [69]. This was shown in the case of the triazines but for other systems suffering from particularly slow mass transfer this may not be the case. Here chiral resolution is observed only at low flow-rates (Fig. 11).

8. New polymerization techniques

As appears from above, MIPs have so far been prepared in the form of continuous blocks that need to be crushed and sieved before use. This results in a low yield of irregular particles, a high consumption of template and a material exhibiting low chromatographic efficiency. There is therefore a need for molecularly imprinted materials that can be prepared in high yield in the form of regularly shaped particles with low size dispersity and a controlled porosity. These are expected to be superior in terms of mass transfer characteristics and sample load capacity compared to the materials obtained from the monolith approach. However, the results obtained so far using alternative approaches, although showing some improvements, have been disappointing.

Bead sized MIPs have been previously prepared through suspension polymerization techniques either using fluorocarbons (Fig. 12) [70] or water [71] as continuous phase, dispersion polymerization or precipitation polymerization [52,72]. This resulted in spherical particles of a narrow size distribution. These procedures have the limitation of being sensi-

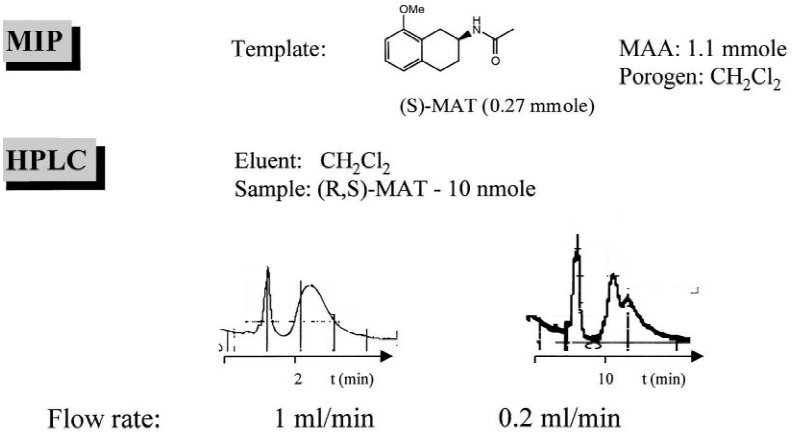


Fig. 11. Elution profiles of (*R*,*S*)-methoxyamidotetralin (MAT) applied on an (*S*)-amidotetralin imprinted stationary phase. Mobile phase: CH_2Cl_2. Sample: (*R*,*S*)-MAT, 10 nmol. Flow-rate: 1 ml/min or 0.2 ml/min. The polymer was prepared using 0.27 mmol template and 1.1 mmol MAA and otherwise as described in Fig. 2. The first eluting peak is due to breakthrough of the solute.

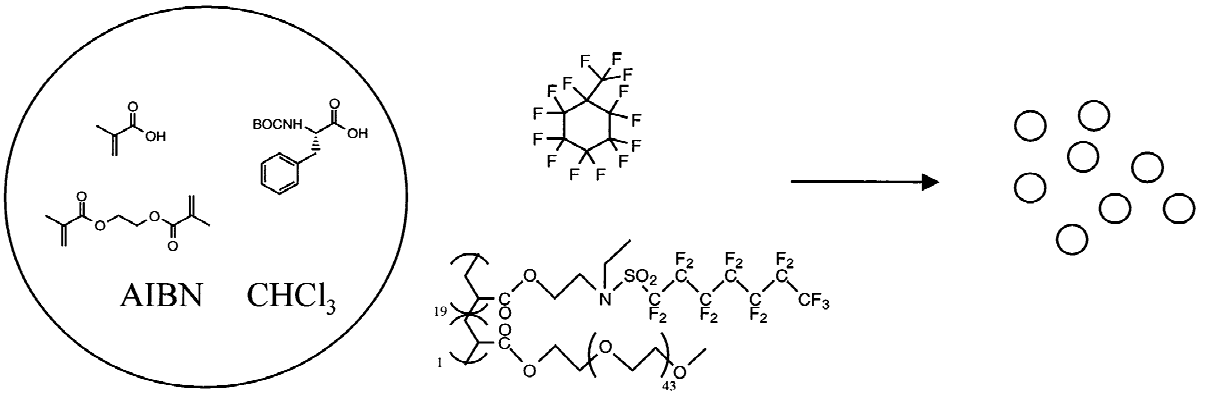


Fig. 12. Suspension polymerization technique for noncovalent imprinting.

tive to small changes in the manufacturing conditions and the type of solvents and polymerization conditions that can be applied but once appropriate conditions have been found they should offer an economic alternative for upscaling. An alternative to this procedure is the coating of preformed support materials [73–75]. MIPs have been prepared as grafted coatings on metaloxide supports [73,75] on organic polymer supports [74] and on the walls of fused-silica capillaries [76–78]. These techniques however involve many steps and are thus associated with larger batch to batch variations. In addition, problems appear in achieving homogenous coatings and to suppress secondary interactions with the support surface.

Much effort has been devoted to the development of a multi-step swelling polymerization technique using water as suspension medium [79]. This has resulted in polymers showing similar selectivities but slightly improved mass transfer characteristics compared with the corresponding monolithic polymers. Of particular relevance for bioanalytical applications was the functionalization of the outer surface of a polymer imprinted with (*S*)-naproxen with a hydrophilic polymer layer (Fig. 13). This led to a slight decrease in the separation efficiency (Fig. 14) but

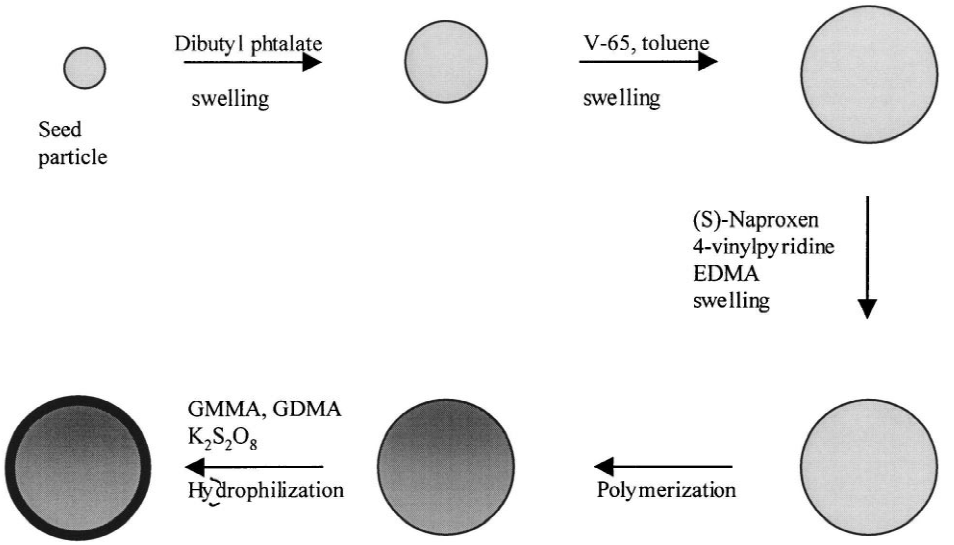


Fig. 13. Synthetic scheme of surface modified MIP for (*S*)-naproxen. V65 = 2,2′-Azobis(2,4-dimethylvaleronitrile), GMMA = glycerolmonomethacrylate, GDMA = glyceroldimethacrylate.

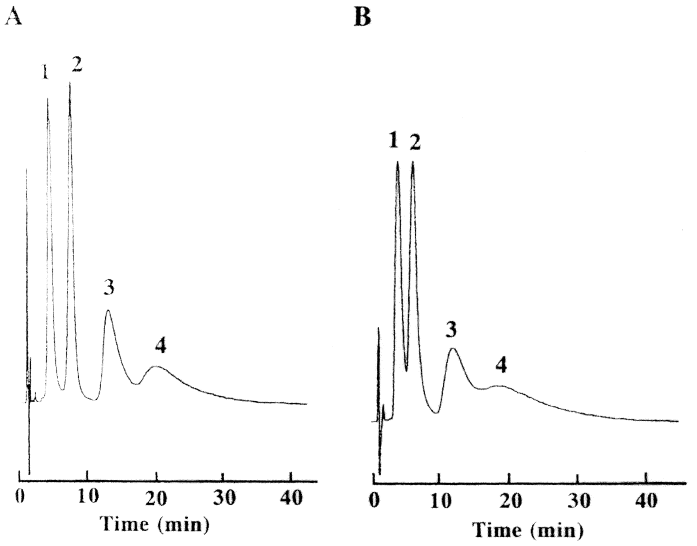


Fig. 14. Elution profile of racemic ketoprofen (1) and ibuprofen (2) and (*R*)- (3) and (*S*)- (4) naproxen on an unmodified (A) and surface modified (B) polymeric packing imprinted with (*S*)-naproxen. Mobile phase: 20 m*M* phosphate buffer (pH 3.2)–MeCN (50:50, v/v). Reproduced from Ref. [79].

allowed on the other hand direct injection of plasma samples on the columns.

9. Mobile phase dependence in chromatography using MIPs

The rebinding to MIPs is strongly dependent on the medium. For predictions of the optimum medium for rebinding, factors related to template structure as well as to polymer structure and morphology have to be considered. In method development involving MIPs the medium used in the rebinding step has therefore to be carefully optimized in order to fully exploit the MIPs ability to recognize the target template. Based on the increasing data available on the dependence of retention and selectivity in various media on the structure of the template, some general rules can be formulated. In the imprinting protocol using MAA as the functional monomer the molecular recognition can be driven by hydrogen bonding, ion exchange and/or the hydrophobic effect depending on the template and the medium.

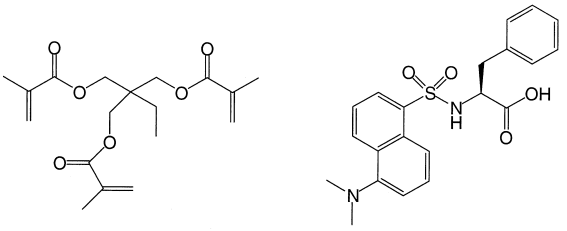
9.1. Retention mechanisms in organic mobile phases

For low to moderately polar templates, good recognition is generally seen using organic solvents as rebinding media where the template interacts mainly electrostatically with the binding sites. In fact, most rebinding experiments to MIPs demonstrating high affinity and selectivity have been performed using organic solvents and for most templates the first chromatographic rebinding evaluation is therefore most often carried out in organic mobile phase systems [80]. A large number of polymers imprinted with *N*-protected amino acids have been prepared and studied for their chromatographic behavior in organic mobile phases [17,19,64,67,81]. In most cases these have been prepared using a poorly hydrogen bonding diluent such as chloroform [67,81] or acetonitrile [19,64] and MAA and/or VPY or AAM as functional monomers. They are then commonly evaluated in media consisting of the same solvent used as diluent together with a polar modifier.

The role of the mobile phase composition using a MIP for BOC-L-phenylalanine was studied by Allender et al. in order to gain a better understanding of the retention mechanism [81]. First of all chloroform, the same solvent used as diluent, was chosen as the base solvent in the mobile phase. In addition to chloroform, nonpolar solvents such as hexane and 2,2,4-trimethylpentane were studied as base solvents. However in spite of longer retention times no or little

selectivity was observed in these phases. The low swelling in such solvents should lead to pore closing and low accessibility to the imprinted sites. The chloroform mobile phase was then modified by the addition of different amounts of polar modifiers. The effect of the modifiers on the retention and selectivity in the separation of both enantiomers on the phase was discussed in terms of the physical properties of the modifier. It was observed that the retention of the enantiomer corresponding to the imprinted one correlated linearly with the hydrogen bond donor parameter taken from the literature (Fig. 15) with tetrahydrofuran (THF) giving the highest selectivities. Since THF has no hydrogen bond donor capacity, this may indicate that modifier interactions involving the hydrogen bond accepting sites of the template or the polymer functional groups negatively affect the recognition. This should be noted also in the context of the solvent memory effect where best recognition often is seen in the same solvent used in imprinting [54,82]. One explanation for this effect is that the sites are complementary for the template solvated in the same solvent used as diluent. Since chloroform is mainly a hydrogen bond donor it is likely to interact mainly with the hydrogen bond accepting groups of the template or the binding sites and therefore, a decreasing tendency of the modifier to compete for these sites will lead to a better recognition.

A recent report by O'Brien et al. support this picture [45]. They performed a detailed chromatographic investigation of a polymer imprinted with dansyl-L-phenylalanine (**23**) using MAA and 2-vinylpyridine as functional monomers. Using acetonitrile containing addition of acetic acid and pyridine as modifiers they found a pronounced influence of the ratio of these modifiers on the retention and selectivity. Thus selectivity increased with increasing ratio acetic acid/pyridine, an effect attributed to a stabilization of an enantioselective interaction between the dansyl-amino group and polymer bound carboxylic acid groups.



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9.2. Retention mechanisms in aqueous mobile phases

As mentioned above, templates with protolytic functional groups, i.e., Brönsted basic or acidic groups are usually successfully imprinted using an acidic (i.e., MAA) or basic (i.e., VPY) functional monomer. The resulting phases often show good chromatographic performance and excellent selectivity in aqueous mobile phases where the retention is driven by ion exchange [13]. This was observed in the mobile phase optimization for the resolution of D,L-PA on an L-PA imprinted polymer [13]. Using polymers imprinted with L-PA and a control polymer imprinted with benzylamine (BA) as stationary phases, Fig. 16 shows that each polymer bound preferentially the compound used as template. The selectivity, reflected in the separation factor α, was high and constant at low pH values whereas when pH_{app} exceeded the pK_a value of the solute, α dropped off to approximately 1 at $pH_{app}=9.5$. The fact that maximum retention was observed at a pH_{app} corresponding to the apparent pK_a of the solute

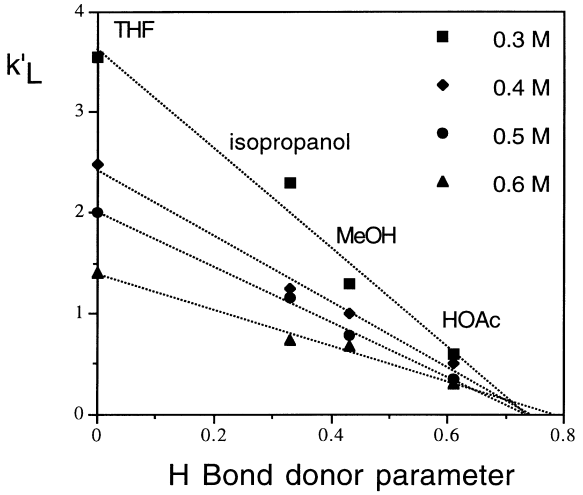

Fig. 15. Influence of hydrogen bond donor number of the mobile phase modifier on the capacity factor (k'_L) of BOC-L-phenylalanine injected (10 μg) on an MIP imprinted with the L-enantiomer. Mobile phase: methylenechloride containing various amounts of the polar modifiers. Reproduced from Allender et al. [81].

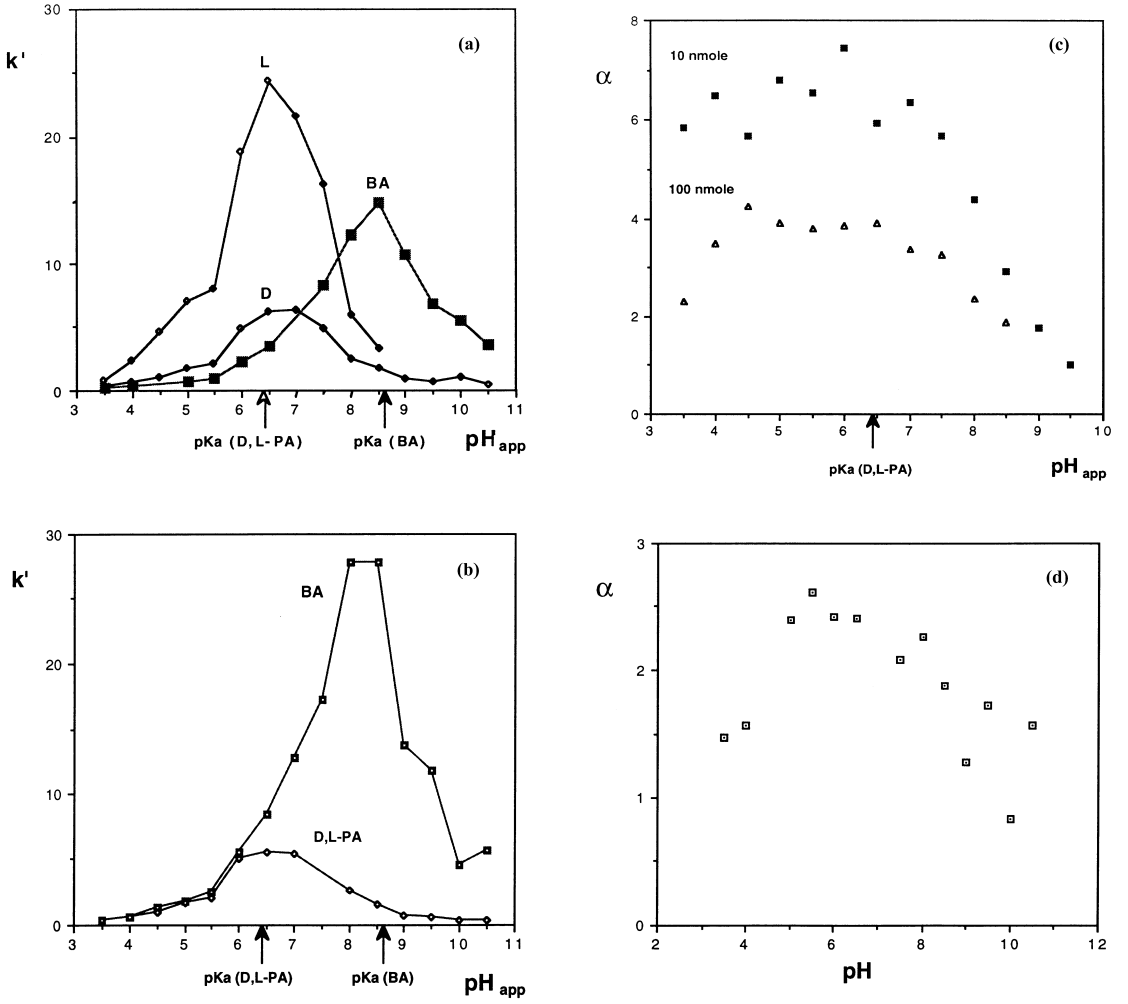

Fig. 16. Retention (k') of D- and L-PA and benzylamine (BA) on (A), an L-PA MIP: PLPA and (B) a MIP imprinted with benzylamine: PBA, injecting 100 nmol solute versus mobile phase apparent pH (pH_{app}). (C) and (D) show the corresponding separation factors (α) obtained at two different sample loads. The separation factor of D,L-PA (C) was calculated as $\alpha = k'_L/k'_D$ and of benzylamine (100 nmol) (D) as $\alpha = k'_{BA}$(on PBA)$/k'_{BA}$(on PLPA). Figure reproduced from Sellergren and Shea [13].

suggested that the retention was controlled by a simple ion-exchange process. Thus the amino group containing solute B (D,L-PA or BA) is bound to the polymer containing carboxylic acid groups (HA) forming ion-pairs BH^+A^-. Assuming that ion exchange is the predominating retention mechanism the capacity factor can be expressed as [83]:

$$k'_B = K\alpha^*{}_B\alpha^*{}_A \quad (4)$$

where $\alpha^*{}_A$ and $\alpha^*{}_B$ are the degree of ionization of the acid (A) and the base (B), respectively and K is a constant for a given column and ionic strength. In order to test the validity of this model, $\alpha^*{}_A$ and $\alpha^*{}_B$ was determined by potentiometric titrations and Eq. (4) plotted as a function of pH_{app}. The agreement with the experimental chromatographic data, both in the pH where the maxima were found and in the relative retention of the solutes at these maxima supported that ion exchange was the primary contribution to the retention.

When increasing the aqueous content in the mo-

bile phase, polar templates become usually less retained on MIPs whereas templates of moderate to low polarity become more retained. The latter increase in retention is due to the hydrophobic effect [37,84]. Thus in contrast to the behavior of other types of affinity phases with biological recognition elements such as immunoaffinity phases [85] the imprinted phases behave more like reversed-phases when the aqueous content is high [48,71,84,86]. This leads to pronounced nonspecific binding often in the form of a total retention of all hydrophobic compounds. However to some extent this nonspecific binding can be reduced by the addition of an organic modifier or a detergent [86].

This has been used in the development of a number of competitive aqueous assays using MIPs often with detection limits and selectivities similar to those reported using corresponding immunoassays [86,87]. Thus many templates respond to an increase in the aqueous content of the mobile phase by showing strong and selective retention at low and high aqueous content [88]. This depends on the hydrophobicity of template substituents as shown in the imprinting of triazines of different hydrophobicities.

In view of the complex retention mechanisms involved in HPLC using MIP phases an extensive mobile phase optimization must thus be carried out for most new templates.

10. Conclusions

A number of conditions will directly influence the development of a new MICSP. The availability of the template in preparative amounts will determine whether it will have to be recycled or a template analogue will have to be used. The latter alternative should also be considered in cases where the template is unstable or poorly soluble in the monomer mixture. Depending on the format of the separation, the polymer must meet certain requirements. If the material is going to be used as a HPLC stationary phase, monodisperse spherical particles are desirable and rapid adsorption–desorption of the template to the sites is necessary for high-performance separations. However, broad and asymmetric band shapes and low saturation capacities due to the heterogeneous distribution of binding sites and slow mass transfer processes are important problems that strongly limit the possible applications of these phases in analytical and preparative chromatography. Nevertheless, with designed functional monomers, new polymerization techniques and combinatorial synthesis and screening techniques MICSPs that meet the above requirements may soon be a reality.

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