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Influence of mobile phase composition and cross-linking density on the enantiomeric recognition properties of molecularly imprinted polymers

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Abstract

A series of experiments were conducted to investigate elements which affect the enantiomeric recognition properties of molecularly imprinted polymers (MIPs) in the HPLC mode. Our results show that the recognition properties of MIPs are greatly influenced by the mobile phase used. For a polymer prepared in acetonitrile, a good enantiomeric separation was observed when acetonitrile-based mobile phase was used, when the mobile phase was changed to chloroform-based, no enantiomeric recognition was observed although the sample molecule was retarded. This indicates that the specific co-operative binding interactions between the functional groups at the imprinted polymer's recognition sites and the sample molecule were considerably disrupted and only non-specific interactions remained. When the mobile phase was changed back to acetonitrile-based, the recognition was regained. In contrast, for polymers prepared in chloroform, chloroform-based mobile phase gave much better separation than acetonitrile-based mobile phase. When other solvents were tested, significant solvent effects were generally observed. Based on these observations, the recognition properties of the methacrylic acid (MAA)–co-ethylene glycol dimethacrylate (EGDMA) polymers were reinvestigated, and the results show that by simply using an optimised mobile phase system, significantly improved recognition over previously reported results was observed. For a polymer made against Cbz-L-Trp, 100 μg of Cbz-D,L-Trp was separated with a separation factor (α) of 4.23 and a resolution (R_s) of 3.87, whereas in the previous report, 10 μg of Cbz-D,L-Trp was only separated with $\alpha=1.67$ and $R_s=0.1$. It is generally realised that the imprinted polymer's recognition property is also very much influenced by the nature of the polymer network. It was shown that the recognition decreased with a decrease in the apparent degree of cross-linking (molar percentage of cross-linker in the polymerisation mixture). Nonetheless, our results show that in our optimised assay system a significant separation could still be obtained on a polymer which was only 22% cross-linked. We consider this to be of importance, since it may suggest a way of imprinting larger molecules because of the possibly improved mass transfer in low cross-linking density polymers. It was reported that when trifunctional cross-linkers [for example: trimethylolpropane trimethacrylate (TRIM)] were used as the cross-linker instead of EGDMA, considerably improved enantiomeric separation and resolving capability were observed. Our results show that the improved performance of the MAA–co-EGDMA MIPs is actually comparable to the performance of the MIPs prepared with those trifunctional cross-linkers. The combination of a hydrogen bonding functional monomer (acrylamide) with TRIM also did not give improved recognition. The results suggest that although the three-dimensional network of these two kinds of polymer may be quite different, the observed recognition improvements were probably largely due to solvent effect.

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1. Introduction

To recognise a certain type of molecule with very high specificity in a complex mixture is one of the basic properties of many bio-molecules. By the specific action of enzymes, receptors, antibodies, etc., a living organism is able to create building blocks from simple precursors, respond to environmental changes, and defend itself against invading substances.

The development of a synthetic approach that could be used to produce materials with comparable recognition properties is of obvious importance. Many such attempts have been made in recent years, and the well-known crown ethers, cryptates, cyclodextrins, concaves and cyclophanes have shown various molecular recognition properties [1–5].

Molecular imprinting technology provides a promising alternative way to create highly specific recognition sites within a synthetic polymer network via the template polymerisation process. Generally, a template molecule is mixed in an organic solvent with a functional monomer (or a combination of several functional monomers), the functional monomer interacts with the functional groups of the template molecule either covalently or non-covalently, the position of the functional monomer is then fixed by co-polymerisation with a cross-linker, which gives a rigid insoluble polymer. The template molecule is removed afterwards by a simple washing (or cleaving) procedure, leaving a three-dimensional porous polymer network with recognition sites which could interact with the template molecule highly specifically [6–9].

Imprinted polymers have been prepared as antibody and receptor mimics [10–14], as sensing materials [15–17], as chromatography stationary phase for separation/purification purposes [18–27], and attempts have also been made to prepare imprinted polymers with certain catalytic properties [28–32].

Various attempts have been made in recent years to improve the recognition performance of imprinted polymers [33–41], which include using different functional monomers, cross-linkers, solvents and polymerisation conditions, etc. The recognition mechanism has also been studied [37,40,42–48].

In our previous studies, we have shown that the enantiomer and substrate selective recognition properties of molecularly imprinted polymers (MIPs) are largely dependent on the size, shape, the physical/chemical properties and relative position of the functional groups of the recognition sites and the sample molecule. In organic media, polar interactions (hydrogen bonding, ionic interactions, etc.) are mainly responsible for the binding and recognition, whereas in aqueous media, hydrophobic interactions play an important role [40,46,47].

In order to gain more insight into the origin of the recognition properties of the imprinted polymers, we have conducted a series of experiments in this investigation concerning the influence of the mobile phase, the cross-linker, the functional monomer, and the apparent degree of cross-linking on the recognition site integrity, and the subsequent recognition properties of the imprinted polymers.

2. Experimental

2.1. Materials

Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were purchased from E. Merck (Darmstadt, Germany). Trimethylolpropane trimethacrylate (TRIM) and methyl methacrylate (MMA) were from Aldrich (Steinheim, Germany). Acrylamide (AA) was obtained from Bio-Rad (Richmond, CA, USA). Chiral compounds were from Sigma (St. Louis, MO, USA), Nova Biochem (Läufelfingen, Switzerland) and Bachem (Bubendorf, Switzerland). 2,2′-Azobisisobutyronitrile (AIBN) was from Janssen (Beerse, Belgium). All organic solvents were of analytical or HPLC grade. Acetonitrile and chloroform were dried over 4-Å molecular sieves before being used for polymer synthesis.

2.2. Polymer preparation

Normally the required amount of template, functional monomer (MAA or AA), cross-linker (EGDMA or TRIM), and initiator (AIBN) were dissolved in chloroform or acetonitrile (Table 1) in glass tubes. They were degassed in a sonicating water bath and saturated with nitrogen for 5 min. The tubes were then sealed with Parafilm and polymerised under UV irradiation (365 nm) at 4°C for 24 h. After the polymerisation had completed, the tubes were carefully broken and the polymers collected and ground in a mechanical mortar. The polymers were wet sieved with acetone through a 25-μm sieve, the particles which passed the sieve were collected, and those of size bigger than 25 μm were dried, ground, and sieved again. This process was repeated several times until all the particles passed through the sieve. The fines were removed by repeated sedimentation in acetone.

When EGDMA was used as the cross-linker, a molar ratio of template to functional monomer to cross-linker of 1 (or 0.5):4:20 was used. When TRIM was used as the cross-linker, in order to make the results comparable, two recipes were used. One was the conventional recipe for preparing TRIM MIPs with a molar ratio of template to functional monomer to cross-linker of 1 (or 0.5):4:4 [39]. In the other recipe, the amount of TRIM was weighted accordingly so that the total quantity of double bonds added to the imprinting mixture equalled to that of EGDMA according to the normal EGDMA recipe, and the molar ratio of template to functional monomer to cross-linker appeared to be 1 (or 0.5):4:13.3.

Because of the limited solubility of the template molecule and the functional monomer in chloroform

Table 1
Polymers prepared in this investigation

Polymer[a]	Print molecule	Amount of print molecule (g)	Functional monomer	Amount of functional monomer (g)	Cross-linker (+MMA)	Amount of cross-linker (g)	Solvent	Amount of solvent (ml)
Pol 1	Boc-L-Trp	0.7676	AA	0.7172	EGDMA	10.00	CH_3CN	15
Pol 2	Boc-L-Trp	2.2823	AA	2.132	TRIM	10.15	CH_3CN	20
Pol 3	Ac-L-Trp-OEt	0.6919	AA	0.7172	EGDMA	10.00	CH_3CN	15
Pol 4	Ac-L-Trp-OEt	0.6919	AA	0.7172	EGDMA	10.00	$CHCl_3$	15
Pol 5	Cbz-L-Ala-NH_2	0.560	AA	0.7172	EGDMA	10.00	CH_3CN	15
Pol 6	Boc-L-Trp	0.7676	AA	0.7172	EGDMA	10.00	$CHCl_3$	15
Pol 7	Boc-L-Trp	0.7676	AA	0.7172	TRIM	11.38	$CHCl_3$	15
Pol 8	Boc-L-Trp	0.7676	MAA	0.8686	EGDMA	10.00	$CHCl_3$	15
Pol 9	Cbz-L-Trp	0.8536	MAA	0.8686	EGDMA	10.00	$CHCl_3$	15
Pol 10	Cbz-L-Glu	0.3548	MAA	0.8686	EGDMA	10.00	$CHCl_3$	15
Pol 11	Boc-L-Trp	0.7676	MAA	0.8686	EGDMA+MMA	8.00+2.00	$CHCl_3$	15
Pol 12	Boc-L-Trp	0.7676	MAA	0.8686	EGDMA+MMA	6.00+4.00	$CHCl_3$	15
Pol 13	Boc-L-Trp	0.7676	MAA	0.8686	EGDMA+MMA	4.00+6.00	$CHCl_3$	15
Pol 14	Cbz-L-Glu	0.3548	MAA	0.8686	TRIM	11.38	$CHCl_3$	15
Pol 15	Cbz-L-Glu	1.0549	MAA	2.5827	TRIM	10.15	$CHCl_3$	20
Pol 16	Boc-L-Trp	0.7676	MAA	0.8686	TRIM	11.38	$CHCl_3$	15
Pol 17	Boc-L-Trp	2.2823	MAA	2.5827	TRIM	10.15	$CHCl_3$	20
Pol 18	Boc-L-Trp	2.2823	MAA	2.5827	TRIM	10.15	$CHCl_3+CH_3CN$	16+4
Pol 19	Cbz-L-Tyr	0.7953	MAA	0.8686	EGDMA	10.00	$CHCl_3$	15
Pol 20	Boc-L-Trp	0.7676	AA	0.7172	TRIM	11.38	CH_3CN	15
Pol 21	Boc-L-Trp	2.2823	AA	2.132	TRIM	10.15	$CHCl_3$	25
Pol 22	Boc-L-Trp	2.2823	AA	2.132	TRIM	10.15	$CHCl_3+CH_3CN$	18+2
Pol 23	Boc-L-Trp	2.2823	AA	2.132	TRIM	10.15	$CHCl_3+CH_3CN$	16+4

[a] Because of the limited solubility of the template and the functional monomer at lower temperature, Pol 21 was prepared via thermal polymerisation at 60°C for 18 h. Mixtures of acetonitrile and chloroform were used to prepare Pol 18, 22, and 23. Pol 22 was prepared at ambient temperature.

at lower temperature, Pol 21 was prepared via thermal polymerisation at 60°C for 18 h. Acetonitrile and chloroform were used in combination for polymer Pol 18, 22 and 23 preparation. Pol 22 was prepared under UV irradiation (365 nm) at ambient temperature for 24 h.

2.3. High-performance liquid chromatography (HPLC)

After sedimentation, polymer particles were slurry packed into 250×4.6 mm HPLC columns at 30 MPa using an air-driven fluid pump and acetone as solvent. The column was washed on-line with 10% HOAc in methanol until a stable baseline was reached. Unless otherwise specified, HPLC analysis was performed at a flow-rate of 1.0 ml/min at ambient temperature and monitored by a UV detector at the maximum absorption wavelength of the analyte. When the mobile phase was changed from one solvent system to another, the column was washed at least 40 min with the new mobile phase before the analysis.

Acetone was used as the void marker. Capacity factors (k'_D and k'_L), separation factor (α) were calculated according to standard chromatographic procedures as $k'_D=(t_D-t_0)/t_0$, $k'_L=(t_L-t_0)/t_0$, $\alpha=k'_L/k'_D$, where t_D is the retention time of the D enantiomer, t_L is the retention time of the L enantiomer, and t_0 is the retention time of the void marker [49,50]. A slightly modified method was used to calculate the resolution (R_s): a straight line was drawn perpendicularly from the peak maximum to the baseline, and the baseline peak band divided into two halves. Since the peaks were asymmetric, only the baseline half band adjacent to the next peak was chosen (its width was referred to as $W_D^{1/2}$ or $W_L^{1/2}$), and the resolution was calculated as $R_s=(t_L-t_D)/(W_D^{1/2}+W_L^{1/2})$.

3. Results and discussion

When the covalent imprinting approach was employed, it was considered that although the porogen used had a strong influence on the morphology of the polymer network, its effect on the selectivity of the recognition sites was not very significant [9], whereas when the non-covalent approach was used, it was shown that a polymer prepared in a relatively non-polar organic solvent performed better than the one prepared in a polar organic solvent. This could be attributed to the fact that relatively non-polar organic solvents favour the formation of the non-covalent interactions between the template molecule and the functional monomer [37,38,40]. The influence of solvents used on the recognition properties of imprinted polymers was also studied. It was suggested that for HPLC analysis, the mobile phase should be chosen similar to the solvent used for the polymer preparation in order to mimic the interactions existing prior to and during the polymerisation [43]. In a recent publication, by comparing the binding affinity of 9-ethyladenine on the corresponding imprinted polymers prepared in different solvents, Spivak et al. showed that the ideal rebinding condition for a given template should include the solvent used as porogen [45]. We also observed that for amide MIPs (MIPs prepared using acrylamide as the functional monomer) made in acetonitrile, the recognition varied substantially when the mobile phase was changed from acetonitrile-based to other organic solvent-based or to water–acetonitrile-based solvent systems [40,46,47].

In this study, the solvent effect on the enantiomeric recognition properties of the imprinted polymers was investigated further in detail. We observed that for the polymers made in acetonitrile, chloroform-based mobile phase gave no separation, although the sample molecule did bind to the polymer. For an AA–co-EGDMA polymer made against Boc-L-Trp (Pol 1) in acetonitrile, a good separation was observed (40 μg of Boc-D,L-Trp; $k'_D=1.09$, $k'_L=3.06$, $\alpha=2.80$, $R_s=2.22$) when 0.1% HOAc in acetonitrile was used as the mobile phase, when the mobile phase was changed to 0.1% HOAc in chloroform, no separation was observed ($k'_D=k'_L=1.63$). When the mobile phase was changed back to 0.1% HOAc in acetonitrile, the separation was regained ($k'_D=1.08$, $k'_L=3.10$, $\alpha=2.88$, $R_s=2.16$). For an AA–co-TRIM polymer prepared against the same template molecule (Pol 2) in acetonitrile, similar results were observed. For an AA–co-EGDMA polymer imprinted against Ac-L-Trp-OEt in acetonitrile (Pol 3), an enantiomeric separation was observed when acetonitrile was used as the mobile phase (0.4 μg of

Ac-D,L-Trp-OEt; k'_D=0.14, k'_L=0.25, α=1.77, R_s=0.49), but when chloroform–heptane (1:1) was used as the mobile phase, no recognition was observed although the sample molecule bound quite strongly to the polymer (k'_D=k'_L=4.09), indicating that the binding was mainly non-specific. For the AA–co-EGDMA polymer made against Ac-L-Trp-OEt in chloroform (Pol 4), the resolution of 40 μg of Ac-D,L-Trp-OEt was improved substantially when the mobile phase was changed from chloroform (α=1.63, R_s=0.54) to chloroform–heptane (1: 1) (α=1.56, R_s=1.70). Also, for an AA–co-EGDMA polymer imprinted against Cbz-L-Ala-NH_2 in acetonitrile (Pol 5), the separation of 40 μg of Cbz-D,L-Ala-NH_2 in acetonitrile (k'_D=0.57, k'_L=0.88, α=1.55, R_s=1.14) was better than the separations in acetonitrile–chloroform (3:1) (k'_D=0.41, k'_L=0.58, α=1.41, R_s=0.70) and in acetonitrile–ethyl acetate (1:3) (k'_D=0.67, k'_L=0.97, α=1.44, R_s=0.83).

We also observed that for the MIPs made in chloroform, the enantiomeric resolutions obtained using chloroform-based mobile phase were much better than those obtained using acetonitrile-based mobile phase. For an AA–co-EGDMA polymer (Pol 6) made against Boc-L-Trp in chloroform, 40 μg of Boc-D,L-Trp could be separated with k'_D=3.59, k'_L=4.81, α=1.34, R_s=1.15 when acetonitrile was used as the mobile phase, whereas 100 μg of Boc-D,L-Trp could be separated with k'_D=2.57, k'_L=4.51, α=1.76, R_s=1.92 when 0.5% HOAc in chloroform was used as the mobile phase. For an AA–co-TRIM polymer (Pol 7) made against the same template molecule in chloroform, 40 μg of Boc-D,L-Trp could be separated with k'_D=3.21, k'_L=4.07, α=1.27, R_s=1.11 when acetonitrile was used as the mobile phase, whereas 100 μg of Boc-D,L-Trp could be separated with k'_D=2.28, k'_L=4.23, α=1.86, R_s=1.90 when 0.5% HOAc in chloroform was used as the mobile phase.

In agreement with previous studies [45], our results indicate that solvents influence the enantiomeric recognition properties of imprinted polymers mainly in two ways: firstly, a solvent with higher polarity competes more efficiently with the sample molecule for the binding of the functional groups of the recognition sites and thus weakens the specific polar interactions, as discussed previously [40,44]. Secondly, it has been shown that, due to the different solvation properties of different solvents for a given type of polymer (Tables 2 and 3), imprinted polymers often exhibit different swelling properties in

Table 2
Enantiomeric separation of Boc-D,L-Trp on MIPs imprinted against Boc-L-Trp using different mobile phases

Polymer	Solvent	Polarity[c]	Dielectric constant[d]	Dipole moment[d] (D)	Solubility parameter $(MPa)^{1/2}$	HBD[e]	HBA[e]	HAc in mobile phase (%)	k'_D	k'_L	α	R_s
Pol 8[a]	$CHCl_3$	4.1	4.81	1.04	19.0	0.15	0.02	0.1	1.59	7.50	4.73	4.54
Pol 1[b]								0.1	2.43	2.43	–	–
Pol 8	CH_2Cl_2[f]	3.1	8.93	1.60	19.8	0.10	0.05	5.0	0.43	1.32	3.06	3.76
Pol 1								1.0	1.20	1.39	1.16	0.09
Pol 8	CH_3COOEt	4.4	6.08	1.78	24.3	0.00	0.45	0.1	0.10	0.26	2.83	1.19
Pol 1								0.1	0.76	1.10	1.46	0.41
Pol 8	CH_3CN	5.8	36.64	3.92	24.3	0.07	0.32	0.1	0.55	0.81	1.46	1.15
Pol 1								0.1	1.20	3.90	3.26	2.24
Pol 8	THF[g]	4.0	7.52	1.75	18.6	0.00	0.48	0.1	–	–	–	–
Pol 1								0.1	–	–	–	–

[a] A 100-μg amount of Boc-D,L-Trp was injected.
[b] A 40-μg amount of Boc-D,L-Trp was injected.
[c] Data were taken from Ref. [49].
[d] Data were taken from Ref. [52].
[e] HBD: Hydrogen-bond donor acidity; HBA: hydrogen-bond acceptor basicity (Ref. [53]).
[f] When 0.1% HAc in CH_2Cl_2 was used as the mobile phase, the sample molecule could not be eluted from the column, when 1.0% HAc in CH_2Cl_2 was used, 100 μg of Boc-D,L-Trp could be separated with k'_D=2.11, k'_L=10.84, α=5.15, R_s=6.91.
[g] A 10-μg amount of the sample molecule could not be separated.

Table 3
Solubility parameters of related polymers in solvents with different hydrogen bonding capacity[a]

Polymer	δ in $[(MPa)^{1/2}]$ Solvent hydrogen bonding		
	Poor	Moderate	Strong
Poly(methacrylic acid) (MAA)	0	20.3	26.0–29.7
Poly(methyl methacrylate) (MMA)	18.2–26.0	17.4–27.2	0
Poly(ethyl methacrylate) (EA)	17.4–22.7	16.0–27.2	19.4–23.3
MMA–EA–AA (45:45:10)	22.7–26.0	18.2–27.0	26.0–29.7
MMA–EA–MAA (40:40:20)	0	18.2–22.1	19.4–29.7

[a] Data were taken from Ref. [54].

different solvents [37], and the various degree of swelling in different solvents may considerably change the morphology of the polymer network, the size, shape and relative positions of the functional groups of the recognition sites which are essential for the recognition.

For the MIPs made in acetonitrile, chloroform-based mobile phase gave no recognition. This could likely be explained that the swelling effect in chloroform disrupted the recognition sites of the polymer. However, it did not seem to leave a permanent effect on the integrity of the recognition sites, since when the mobile phase was changed back to acetonitrile-based, the recognition was regained. For the MIPs made in chloroform, chloroform-based mobile phase gave better recognition than acetonitrile-based mobile phase could be attributed to the fact that acetonitrile is more polar than chloroform.

Our results also show that for the MIPs made in acetonitrile, although no enantiomeric recognition was observed in chloroform, the sample molecule did bind to the polymer (in some cases quite strongly) because of the non-specific interactions. This suggests that care should be taken when binding parameters (the capacity factor, binding constant, etc.) are used as the proof of specific interactions. To distinguish from the non-specific interactions, blank polymers were sometimes introduced [51], but this does not always solve the problem, since the existence of a template molecule would most likely change the morphology of the polymer network and the distribution of the functional groups, making it difficult to compare the results.

The solvent effect was investigated further using two polymers imprinted against Boc-L-Trp; one was made in chloroform (Pol 8) and the other made in acetonitrile (Pol 1) (Table 2). For the one made in chloroform (Pol 8), 100 μg of Boc-D,L-Trp could be nicely separated in chloroform and dichloromethane, poorer separations were observed when ethyl acetate and acetonitrile were used as the mobile phase, and no separation was observed in tetrahydrofuran (THF). The good separation in dichloromethane could be attributed to its similar structure and polarity to chloroform. Acetonitrile is much more polar than chloroform, so the separation decreased considerably. Ethyl acetate and THF are less polar than acetonitrile and more polar than chloroform. When ethyl acetate was used as the mobile phase, the separation was better than when acetonitrile was used, and much worse than when chloroform and dichloromethane were used. Although ethyl acetate is a much stronger hydrogen bonding acceptor, its overall polarity does not differ very much from chloroform and dichloromethane, so besides the polarity effect, the results suggest that there might also be some structural changes of the recognition sites due to the swelling effect. This is supported by the fact that for a polymer made against Cbz-L-Ala-NH_2 in acetonitrile (Pol 5), when acetonitrile–ethyl acetate (1:3) was used instead of acetonitrile as the mobile phase, the capacity factors increased but the separation decreased. When THF was used as the mobile phase, the recognition disappeared completely, and the sample molecule was not even retarded. This could likely be explained that the swelling effect played a major role. The considerable structural changes of the polymer network made the sample molecule inaccessible to the functional groups of the polymer. For the polymer prepared against Boc-L-Trp in acetonitrile (Pol 1), Boc-D,L-Trp could be nicely separated in acetonitrile, but it could hardly be

separated in chloroform, dichloromethane, THF, and only be moderately separated in ethyl acetate. This suggests that the polymer prepared in acetonitrile is probably more susceptible to the solvation effect. When the mobile phase was changed back to acetonitrile-based, the recognition was regained.

It should also be mentioned that an increase in solvent polarity does not always result in a decrease in recognition. We have observed that for the amide MIPs made in acetonitrile, when water was added gradually to the mobile phase (acetonitrile), the recognition decreased considerably at the beginning, and at a certain point it disappeared completely, but when the amount of water was increased further, the recognition was regained, and it increased with the increase of the water content in the mobile phase. We ascribed this regained recognition to the increased specific hydrophobic interactions between the sample molecule and the recognition sites of the imprinted polymer [46,47].

On the basis of the above observations, we decided to reinvestigate some of the early results obtained with MAA–co-EGDMA MIPs. Significantly improved enantiomeric separations were observed simply when an optimised assay mobile phase system was used. For the polymer imprinted against Boc-L-Trp (Pol 8), a baseline separation of 100 μg of Boc-D,L-Trp was obtained with $\alpha=4.73$ and $R_s=4.54$ (mobile phase: 0.1% HOAc in chloroform), whereas in the previous report, 10 μg of Boc-D,L-Trp was only separated with $\alpha=1.90$ and $R_s=0.8$ (mobile phase: CH_3CN–$CHCl_3$–HOAc, 90:9.5:0.5) [18]. For the Cbz-L-Trp imprinted polymer (Pol 9), the separation was improved from 1.67 to 4.23, and the resolution improved from 0.1 to 3.87. For the Cbz-L-Tyr imprinted polymer, similar improvements were also observed [separation (α) improved from 1.67 to 4.23 and resolution (R_s) improved from 0.1 to 3.87]. For the polymer made against Cbz-L-Glu (Pol 10), the separation and resolution were both improved (separation improved from 2.45 to 2.70, and resolution improved from 1.4 to 2.43), and the load capacity was also increased 10 times.

The influence of the polymer chain mobility on the recognition site integrity was also investigated. This was done by changing the apparent degree of cross-linking. It is considered that at higher degrees of cross-linking, the polymer chain is less mobile and thus helps to retain the recognition site integrity. It has been shown that when the covalent approach was employed, decreasing the apparent degree of cross-linking resulted in a decrease in recognition [9,33]. For the non-covalent approach, the relationship between the polymer's degree of cross-linking and its recognition property has not been well studied. In a few cases, the apparent degree of cross-linking was reduced by increasing the molar ratio of functional monomer to cross-linker. In one such case, it was observed that with a decrease in the apparent degree of cross-linking, the separation increased to a maximum at about 75% cross-linking, followed by a slow decrease upon further lowering of the apparent degree of cross-linking until 50%, where almost all the recognition was lost suddenly [35]. In another case, it was observed that when the apparent degree of cross-linking decreased from 75 to 62.5%, the separation increased [21]. It is therefore not quite clear in these two cases whether the observed effects were due to a change in the number of binding sites (because of the variation of the amount of functional monomer used), or due to a change in the polymer's morphology. In this investigation, a series of polymers with different apparent degree of cross-linking were prepared by adding different amounts of MMA to the imprinting mixture (Table 4). Our results show that the separation decreased with a decrease in cross-linking, as expected, but this effect seemed to be less dramatic than the results obtained previously [33]. For a polymer (Pol 13) prepared with an apparent degree of cross-linking as low as 22.4%, a near baseline separation was still obtained, which suggests that a considerable number of the recognition sites still remained intact. In addition, when Pol 8 was used as the stationary phase, the plate numbers for the D and L enantiomers were 334 and 20, respectively, whereas when Pol 13 was used, these values were 342 and 65, respectively, which suggests that the mass transfer in the low cross-linking density polymer was improved (Figs. 1 and 2).

It is also interesting to note that for the 37.7% cross-linked polymer (Pol 12), 100 μg of Boc-D,L-Trp could be separated with $\alpha=2.33$, $R_s=2.93$ when 0.1% HOAc in $CHCl_3$ was used as the mobile phase, when the mobile phase was changed to 0.1% HOAc in $CHCl_3$–heptane (8:2), an increase in recognition was observed, the same sample could be separated

Table 4
Enantiomeric separation of 100 μg of Boc-D,L-Trp on MIPs prepared against Boc-L-Trp with different degree of cross-linking[a]

MIP	Degree of cross-linking (%)	k'_D	k'_L	α	R_s
Pol 8	83.3	1.59	7.50	4.73	4.54
Pol 11	57.3	1.43	5.56	3.90	3.83
Pol 12	37.7	1.28	2.99	2.33	2.93
Pol 13	22.4	1.35	2.54	1.87	1.67[b]

[a] Mobile phase: 0.1% HAc in $CHCl_3$.
[b] A 10-μg sample was injected.

with $\alpha=2.40$, $R_s=3.42$. For the 22.4% cross-linked polymer (Pol 13), when the same mobile phase change was made, a decrease in recognition was observed, which suggests that the less cross-linked polymer network is probably more susceptible to the swelling effect.

In order to improve the recognition performance of imprinted polymer, a new class of polymer has been synthesised using a number of trifunctional cross-linkers [for example: TRIM, pentaerythritol triacrylate (PETRA)] instead of EGDMA as the cross-linker [39]. The MAA–co-TRIM and MAA–co-PETRA MIPs showed excellent enantiomeric recognition properties and much improved loading capacity. Frontal chromatography analysis showed that the binding constants, as well as the number of binding sites for the same sample molecule, were similar for MAA–co-EGDMA and MAA–co-TRIM MIPs, but the MAA–co-TRIM MIPs seemed to have relatively larger average pore size and pore volume. It was suggested that because of the polyclonal nature of the recognition sites, the MAA–co-TRIM and MAA–co-PETRA MIPs may contain more specific recognition sites, and the relatively more open structure may facilitate mass transfer. Our results show that the improved performance of MAA–co-EGDMA MIPs is actually comparable to that of MAA–co-TRIM and MAA–co-PETRA MIPs (Table 5), which indicates that the structural differences between these two kinds of imprinted polymer probably do not influence their recognition properties as significantly as had previously been believed. The combination of acrylamide and TRIM also failed to give polymers with noticeably improved recognition properties (Table 6).

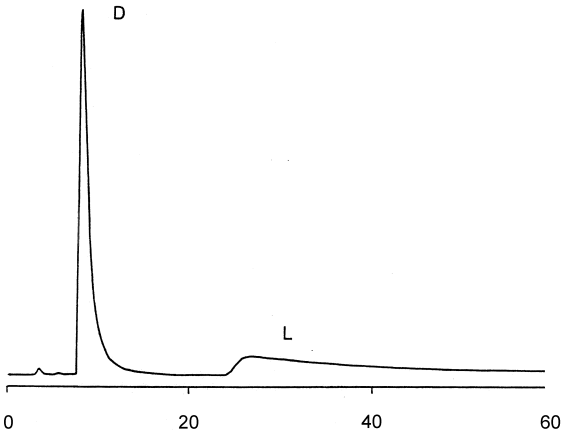

Fig. 1. Enantiomeric separation of 100 μg of Boc-D,L-Trp on a polymer imprinted against Boc-L-Trp (Pol 8). Mobile phase, 0.1% HOAc in chloroform; flow-rate, 1.0 ml/min; detection wavelength, 280 nm; $k'_D=1.59$, $k'_L=7.50$, $\alpha=4.73$, $R_s=4.54$.

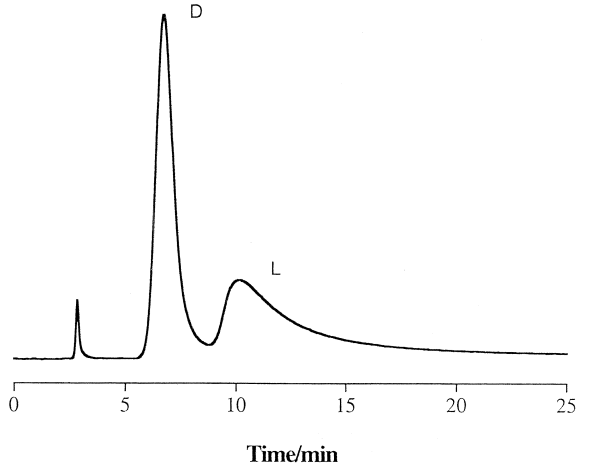

Fig. 2. Enantiomeric separation of 10 μg of Boc-D,L-Trp on a polymer imprinted against Boc-L-Trp (Pol 13). Mobile phase, 0.1% HOAc in chloroform; flow-rate, 1.0 ml/min; detection wavelength, 280 nm; $k'_D=1.35$, $k'_L=2.54$, $\alpha=1.87$, $R_s=1.67$.

Table 5
Comparison of the enantiomeric separations of the corresponding racemates on MIPs prepared using EGDMA, TRIM or PETRA as the cross-linker

Polymer[a]	Template	Cross-linker	Molar ratio[b]	Mobile phase	k'_D	k'_L	α	R_s
Pol 10	Cbz-L-Glu	EGDMA	0.5:4:20	1.0% HAc in $CHCl_3$	1.74	4.70	2.70	2.43
Pol 14		TRIM	0.5:4:13.3	1.0% HAc in $CHCl_3$	1.93	4.75	2.46	2.17
Pol 15		TRIM	0.5:4:4	3.0% HAc in $CHCl_3$	1.97	5.18	2.64	2.63
Ref. [39]		TRIM	0.5:4:4	Gradient eluent[d]	1.77	4.34	2.45	3.10
Pol 8	Boc-L-Trp	EGDMA	1:4:20	0.1% HAc in $CHCl_3$	1.59	7.50	4.73	4.54
Pol 16		TRIM	1:4:13.3	0.1% HAc in $CHCl_3$	1.92	10.34	5.38	4.81
Pol 17		TRIM	1:4:4	1.0% HAc in $CHCl_3$	2.60	14.32	5.52	4.72
Pol 18[c]		TRIM	1:4:4	1.0% HAc in $CHCl_3$	2.14	10.36	4.85	4.35
Pol 19	Cbz-L-Tyr	EGDMA	1:4:20	0.5% HAc in $CHCl_3$	2.04	6.33	3.11	3.05
Ref. [39][e]		PETRA	1:4:4	Gradient eluent	2.32	6.63	2.86	5.47

[a] Polymers were all made in chloroform, methacrylic acid was used as the functional monomer.
[b] The molar ratio refers to template/functional monomer/cross-linker.
[c] This polymer was prepared using a solvent mixture (16 ml $CHCl_3$+4 ml CH_3CN).
[d] A gradient eluent normally results in an increase in resolution and a decrease in separation, see Ref. [40].
[e] Data were taken from Ref. [39].

Table 6
Enantiomeric separation of Boc-D,L-Trp on MIPs prepared using Boc-L-Trp as the template, acrylamide as the functional monomer, and TRIM as the cross-linker

Polymer[a]	Molar ratio[d]	Solvent	Mobile phase	k'_D	k'_L	α	R_s
Pol 2[b]	1:4:4	CH_3CN	0.5% HAc in CH_3CN	2.81	6.77	2.41	1.60
Pol 7[b]	1:4:13.3	$CHCl_3$	0.5% HAc in $CHCl_3$	2.43	5.55	2.28	2.25
Pol 20[b]	1:4:13.3	CH_3CN	CH_3CN	1.85	6.01	3.26	1.83
Pol 21[b]	1:4:4	$CHCl_3$	CH_3CN	6.18	6.95	1.12	0.41
Pol 22[c]	1:4:4	$CHCl_3$+CH_3CN (18+2)	1.0% HAc in CH_3CN	4.25	5.67	1.33	0.95
Pol 23[c]	1:4:4	$CHCl_3$+CH_3CN (16+4)	1.0% HAc; 19% CH_3CN; 80% $CHCl_3$	2.25	2.92	1.30	0.85

[a] Pol 21 was made via thermal polymerisation at 60°C for 18 h, mixtures of acetonitrile and chloroform were used to prepare Pols 22 and 23. Pol 22 was prepared at ambient temperature.
[b] A 40-μg sample was injected.
[c] A 100-μg sample was injected.
[d] The molar ratio refers to template/functional monomer/cross-linker.

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