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Synthetic Peptide Receptor Mimics: Highly Stereoselective Recognition in Non-Covalent Molecularly Imprinted Polymers

Olof Ramström, Ian A. Nicholls and Klaus Mosbach*

Department of Pure and Applied Biochemistry, Chemical Centre, University of Lund, Box 124, S-221 00 Lund, Sweden

Abstract: Ligand-receptor recognition has been studied in a molecularly imprinted polymer (MIP) prepared against *N*-Ac-L-Phe-L-Trp-OMe. The non-ionic non-covalent interaction based recognition was evaluated using the polymers as chiral HPLC stationary phases. Marked regio-, enantio- and diastereo- ligand selectivity were demonstrated with enantiomer separation factors of up to 17.8. The interaction of a series of related structures with the MIP receptor site has provided insight concerning its nature and the recognition mechanisms.

INTRODUCTION

Ligand selective recognition by non-covalent molecularly imprinted polymers[1] (MIPs) has found use in a diverse array of applications ranging from the preparation of artificial antibody combining site mimics[2] and chiral chromatographic stationary phases[3], to the mediation of organic syntheses[4] and as enzyme mimics[5]. The selectivity of these materials for a predetermined ligand makes them versatile systems for the study of molecular recognition phenomena[6]. MIPs constitute an interesting complement to other rationally designed small molecule recognition systems[7].

The principles underlying the preparation of non-covalent molecularly imprinted polymers have been discussed in detail elsewhere[1], but may be summarised as follows, Fig. 1. A monomer or monomer mixture, judiciously selected to ensure chemical functionality complementary to that of the imprint species, is mixed with the imprint molecule in the presence of a suitable cross linking agent. The complementarily interacting functionalities form predictable solution structures[8] which, after polymerisation and extraction of the imprint species, lead to the defining of a recognition site of complementary steric and functional topography to the imprint molecule. Subsequent incubation of a mixture of the imprint molecule and related chemical species results in the selective rebinding of the imprint structure. This selectivity has been utilised in molecularly imprinted polymer based chiral stationary phases (CSPs) which are distinguished from other CSPs by their predictable order of elution, such that application of a racemate of the imprint species always results in longer retention times for the imprinted enantiomer. This effect is reflected by the differing binding affinities of enantiomers at MIP recognition sites[9].

In this study we report the preparation of receptor mimics selective for the dipeptide *N*-Ac-L-Phe-L-Trp-OMe, **1**, by the molecular imprinting of **1** in a methacrylic acid (MAA) (**5**) - ethylene glycol dimethacrylate (EGDMA) (**6**) copolymer and an analysis of molecular recognition in the *anti*-peptide polymer which was undertaken by using it as a CSP. The interactions defining the imprint molecule binding in this *anti*-peptide polymer are non-ionic. Marked enantiomeric resolution, of an order only previously observed in systems utilising ionic interactions, was observed, along with significant diastereo- and regio-selectivity. Furthermore, recognition of partial imprint species structures was demonstrated through a study using a number of structures closely related to parts of **1**. Analysis of the enantio-, diastereo- and regio-recognition phenomena has provided valuable insight into the nature of this artificial receptor site.

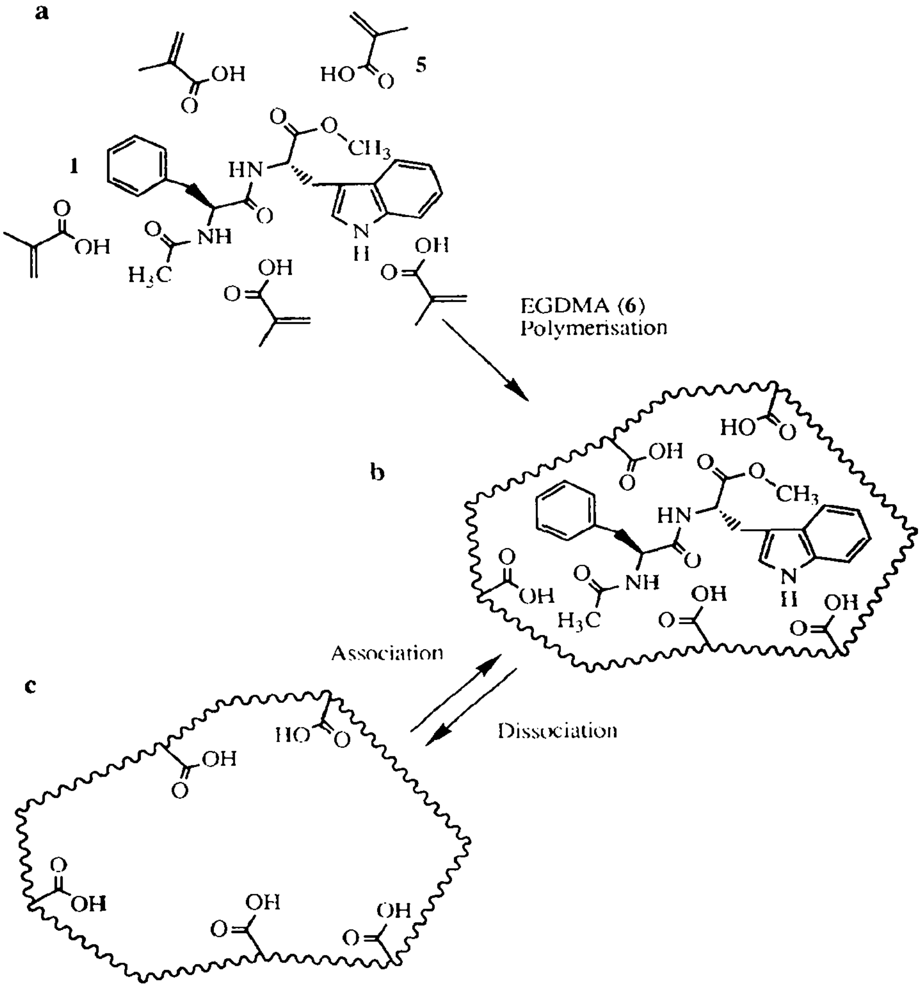
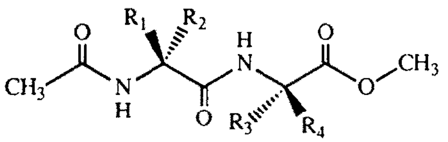


Fig. 1. Schematic representation of the molecular imprinting of *N*-Ac-L-Phe-L-Trp-OMe (**1**) in a methacrylic acid (**5**) - ethylene glycol dimethacrylate (**6**) copolymer. **a**. Hydrogen-bonding interactions between imprint species and functional monomers lead to solution adducts. **b**. Polymerisation captures the topographical relationship present in the solution adduct. **c**. Extraction of the imprint species yields a recognition site of complementary shape and functional topography.

RESULTS

N-Ac-L-Phe-L-Trp-OMe, **1**, was used as an imprint molecule (or template) for preparing the corresponding *anti*-peptide MIP. The imprinted MAA (**5**) - EGDMA (**6**) copolymer was prepared using a 1(**1**):6(**5**):30(**6**) polymerisation mixture ratio, which was shown to yield optimal selectivity, as determined by subsequent HPLC analysis, relative to other ratios of **1**:5. The functional monomer, MAA (**5**), is capable of acting as both a hydrogen bond donor and acceptor. The bulk polymer was processed to render it for use as a CSP for HPLC, packed into HPLC columns, then washed exhaustively under acidic conditions to remove residual print molecule. Combustion analysis data indicated a nitrogen content, in washed and dried polymer samples, consistant with extraction of ≥ 95% of the print species. It was assumed that any residual print molecule was inaccessible to bulk solvent.



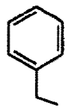
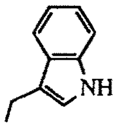
			H-
1	R_1	R_3	R_2 & R_4
2	R_2	R_4	R_1 & R_3
3	R_1	R_4	R_2 & R_3
4	R_2	R_3	R_1 & R_4

Table 1. *N*-Ac-Phe-Trp-OMe Stereoisomeric Recognition by the *Anti*-*N*-Ac-L-Phe-L-Trp-OMe (**1**) MIP.

Stereoisomeric Pair (a-b)	k'_a	k'_b	f/g	α'	α
DD (**2**) - LL (**1**)	0.36	6.40	1.0	16.4	17.8
DL (**4**) - LL (**1**)	0.44	6.20	0.9	13.5	14.2
LD (**3**) - LL (**1**)	1.06	5.51	0.8	5.80	5.21
DL (**4**) - LD (**3**)	0.45	0.98	0.5	2.33	2.20
DD (**2**) - LD (**3**)	0.38	1.03	0.7	2.83	2.75
DD (**2**) - DL (**4**)	0.38	0.38	0.0	1.21	1.00

Chromatographic retention factors (k') and separation factors (α) for the separation of enantiomeric and diastereomeric forms of *N*-Ac-Phe-Trp-OMe (10μg of dipeptide mixtures were injected on each run). Analyses were run in $CHCl_3$/AcOH (99:1). *Effective* enantio-separation values (α') represent separation factors calculated from individual enantiomer sample injections (5μg), where the k' values determined were: LL, 6.15; LD, 1.06; DL, 0.45; DD, 0.38. f/g resolution factors (see reference 16).

The MIP's capacity for resolution of the imprint species (**1**) from its enantiomer, *N*-Ac-D-Phe-D-Trp-OMe (**2**) and its diastereoisomers, *N*-Ac-L-Phe-D-Trp-OMe (**3**) and *N*-Ac-D-Phe-L-Trp-OMe (**4**), was examined, Table 1. Chloroform, the solvent of polymerisation, was used for examining ligand affinity for the recognition sites. This permits the binding to take place under conditions as close as possible to those used during the polymerization process. Acetic acid (≈1%) was incorporated into the eluent mixtures to maintain polymer carboxylic acid residues in their protonated form and so preclude ion-dipole interactions between the polymer carboxylates and the ligands. This was manifested as a sharpening of peak shape corresponding to a reduction in non-specific binding modes. Analysis using a "blank" polymer, prepared in the absence of the imprint species, afforded no evidence of enantiomeric differentiation in any instance.

A range of chiral compounds representing partial imprint molecule structures, tryptophan and phenylalanine derivatives, were examined to probe the recognition site, Table 2. In some instances solvent variations were required to permit solubilisation of the test substances or to optimise recognition. It is again noteworthy that analysis using the "blank" polymer afforded no evidence of enantiomeric differentiation.

Table 2 Chiral Recognition of Partial Imprint Molecule Structures.

Ligand$^{\text{eluent}}$	*Effective* Resolution			Racemic Resolution		
	k'_L	k'_D	α'	k'_L	k'_D	α
H-Trp-OMe (**9**)^a	7.91	7.69	1.03	7.03	7.03	1.0
N-Ac-Trp-OMe (**11**)^b	1.71	1.34	1.28	1.53	1.33	1.15
N-Ac-Trp-OEt (**15**)^c	3.43	2.91	1.18	2.98	2.98	1.0
N-Ac-Trp-NH_2 (**12**)^a	8.04	n.d.	n.d.	8.00	6.17	1.30
N-Ac-Trp-OH (**7**)^a	2.50	2.32	1.08	2.29	2.29	1.0
H-Phe-OMe (**10**)^a	1.39	1.36	1.03	1.27	1.27	1.0
N-Ac-Phe-OMe (**13**)^d	2.49	2.40	1.04	2.44	2.44	1.0
N-Ac-Phe-OEt (**16**)^d	1.79	1.70	1.05	1.74	1.74	1.0
N-Ac-Phe-NH_2 (**14**)^a	2.61	1.52	1.72	2.53	1.49	1.70
N-Ac-Phe-OH (**8**)^b	1.30	1.21	1.08	1.25	1.25	1.0

Injection samples of 5 μg and 10 μg were used for determination of "effective" and racemic separations, respectively. Eluents: a. $CHCl_3$, 1% HOAc (v/v), b. $CHCl_3$/heptane/HOAc 80/20/1 (v/v/v), c. $CHCl_3$/heptane/HOAc 50/50/1 (v/v/v), d $CHCl_3$/heptane/HOAc 25/75/1 (v/v/v). n.d. denotes not determined.

Table 3. Polymer Physical Analyses. *a*. Single Point Gas Adsorption Data. *b*. Solvent Frontal Zone Analysis of the *Anti N* Acetyl L-Phe-L-Trp-OMe MIP.

a.

Polymer	Surface Area (m^2g^{-1})	Pore Size (Å)
MIP	194.6 ± 0.1	80.4 ± 0.1
Blank	292.0 ± 0.1	96.3 ± 0.1

b.

Ligand	K_{diss}/mM	L_t/μmol g^{-1}
LL (**1**)	1.64 ± 0.07	20.8 ± 0.7
DD (**2**)	2.00 ± 0.09	18.7 ± 0.7

a. Surface areas (m^2g^{-1}) and pore diameters (Å) from BET single point gas adsorbtion. *b*. Imprint species **1** and enantiomer, **2**, dissociation constants (K_{diss}) and site population densities (L_t) from solvent frontal analysis on MIP, flow rates of 0.2 mL min^{-1} were employed .

The surface areas of the *anti*-peptide polymer and the non-imprinted "blank" polymer were determined by the single point surface adsorption of gas, Table 3a. These values are in accordance with previous studies on imprinted polymer systems[10,11]. Solvent frontal zone analysis[9], Table 3b, revealed differing MIP binding affinities for the print species (**1**) and its enantiomer, (**2**) and provided an estimate of the number of sites for **1** and **2**.

DISCUSSION

Recognition of the imprint species, the LL dipeptide (**1**), by the *anti*-peptide polymer was examined by comparison with its enantiomeric, DD (**2**), and diastereomeric, LD (**3**) and DL (**4**), forms, Table 1. As expected, the print species was the most strongly retained. The racemate was resolved, with base line separation, separation factor (α-value) 17.8 (Fig. 2), which is significantly greater than any previously reported non-covalent MIP-ligand enantio-recognition[1b]. MIP derived CSPs prepared using ionic interactions to assist in defining the recognition site have been reported to exhibit enantiomeric separation factors of up to 15 [12]. Purely non-ionic interaction based imprinted polymers have, however, been characterised by consistantly lower enantio-recognition properties, α-values of up to 3.9 [3b]. Most interestingly, the diastereomeric dipeptides **3** and **4** were also readily resolved from **1**, again with substantial separation factors and resolutions.

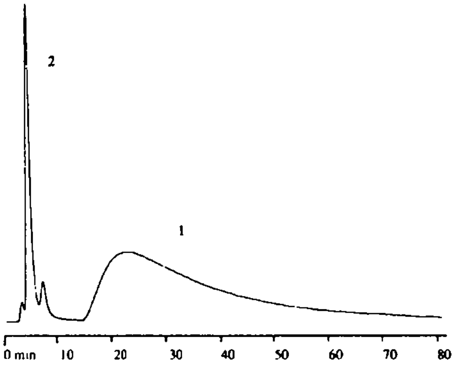


Fig. 2. Chromatogram of the resolution of the print species, Ac-L-Phe-L-Trp-OMe (**1**) from its enantiomer, Ac-D-Phe-D-Trp-OMe (**2**). Attenuation was increased 10-fold after 13 minutes to provide a more clear view of the peak of **1**.

Although the limit of resolution available on this column system failed to effect separation of mixtures of the LD (**3**) and DL (**4**) stereoisomers, careful examination of the retention times of the individual components showed reproducible differences in their affinities, k′ values, allowing calculation of an *effective* separation factor, α'. That **2**, **3**, and **4** bind on the whole is a reflection of the heterogeneity of the receptor site population, analogy may possibly be drawn here to the recognition site distribution present in a polyclonal antibody sample. Undoubtedly, all sites do not possess the same array of interactions and steric complementarity, as illustrated by the broadness of the print species chromatographic response, thus conclusions reflect upon the nature of the "average site" rather than in terms of a uniform site population. The rank order of dipeptide affinities may possibly be explained in terms of differing steric requirements for the recognition of the phenylalanine and tryptophan side chains. A D-configuration at the phenylalanine residue produces a greater reduction in recognition than the presence of a D-configuration at the tryptophan residue. The L-Trp binding domains should more readily accomodate the L-Phe side chains than an L-Phe binding domain can accomodate the more sterically demanding L-Trp side chain. This would equate to a greater opportunity for L-configuration selective binding for the phenylalanine residues than for those of tryptophan, as reflected in the observed relative affinities.

Dissociation constants determined for the print species (**1**) and its enantiomer (**2**) by solvent frontal zone analysis[9], Table 3b, are indicative of the average specificity of the polymer receptor site population. This analysis afforded higher effective site populations for the print species (**1**) than for its optical antipode (**2**) in the MIP. Furthermore, this technique provided a means of determining the binding site populations for the individual enantiomers. The difference between these two values provides an estimate of the number of sites specific for **1**, *i.e.* 2.1 μmoles per gram (dry weight) of polymer.

To further probe the structural and functional features responsible for recognition, parts of the print species were examined as ligands, Table 2. The enantiomer dependent k′ values obtained for their respective *N*-acetyl derivatives (**7** and **8**) and methyl esters (**9** and **10**), however, showed some, albeit weak, enantioselective recognition. Again, the examination of individual enantiomer retention characteristics proved useful. It was considered that strong polar interactions between the ligand amino and carboxyl moieties and the polymer matrix bound carboxyl residues may enhance non-specific binding modes. This contention was supported by the ready resolution of racemic mixtures of *N*-acetyl tryptophan methyl ester (**11**) and *N*-acetyl tryptophanamide (**12**), each without the possibility of ionic interactions. Parallel studies using *N*-acetyl phenylalanine methyl ester (**13**) and amide (**14**) reflected these results. The tryptophan derivatives showed greater non-specific binding, longer retention times, than their phenylalanine counterparts, implicating the extra hydrogen bonding available through the indole nitrogen as contributing to recognition.

Interestingly, *N*-acetyl tryptophan ethyl ester (**15**) was less well resolved than its methyl analogue (**11**), even under carefully optimised elution conditions. This implied the presence of steric constraints in the vicinity of the ligand ester moiety binding domain within the recognition site. Subsequent analysis of *N*-acetyl phenylanlanine ethyl ester (**16**) showed enantiomeric differentiation comparable to the corresponding methyl derivative (**13**). That **13** and **16** are similarly recognised indicates that the spatial constraints within the recognition site better accomodate the ethyl ester moiety of phenylalanine than in the case of the tryptophan derivative (**15**). In the case of **16**, the ethyl group should occupy a spatial domain defined by the bulky -L-Trp-OMe of the imprint species, thus the average site should not possess adverse steric interactions in this region. A further implication of these data is that the receptor site preferably recognises the partial imprint structures in separate binding domains. Previous work using single amino acid derivative based MIPs[1b,3c] has demonstrated some degree of steric latitude with respect to side chain substitution, especially where the groups have been predominantly hydrophobic in character. Collectively, these facts suggest that the more polarised portions of the print molecules, *e.g.* in the vicinity of the ester functionality, relative to more hydrophobic regions, result in a better defined polymeric recognition structure. These observations therefore provide support for and extend the concept of the *prearrangement* of functional monomer units prior to polymerisation[8], whereby the polar functional monomer species interact most strongly with complementarily functionalised binding groups in the print molecule. The stronger the interaction, the shorter the average distance between the two interacting species (Coulomb's Law) in the solution adduct, resulting in a better defined MIP receptor in regions complementary to imprint molecule polar sites. The data presented here imply a fine balance between steric and electronic effects in regulating the MIP-ligand binding event.

CONCLUSION

Molecular imprinting offers a means of constructing recognition sites of predetermined selectivity and is thus an extremely useful tool for the study of molecular recognition phenomena. The levels of "substrate"- and enantioselectivity demonstrated by this *anti*-*N*-acetyl-L-Phe-L-Trp-OMe MIP have previously only been observed for systems utilising ionic interaction facilitated recognition. The dipeptide receptors have been used to investigate factors influencing recognition in methacrylic acid - ethylene glycol dimethacrylate MIPs. Under the rebinding conditions employed, polymer based steric demands have been shown to be more stringent around print molecule site domains corresponding to polarised functionality in the print species. This is a direct consequence of the *prearrangement* phase, when self-assembly of the print molecule-monomer(s) solution adducts takes place, prior to the intitation of polymerisation. MIPs lend themselves to the study of ligand binding phenomena, *e.g.* intrinsic binding constants, rotor freezing, *etc.*, following the principles enunciated elsewhere[6,13]. Finally, MIP tailor-made recognition systems are now capable of racemate resolution on par with the best commercially available CSPs and possess remarkable chemical and mechanical stability, as demonstrated by their repeated use, many hundreds of runs, over several months.

EXPERIMENTAL

General methods.

Reagents and chemicals (Merck, Sigma, Aldrich, Nova Biochem or Bachem AG), initiator (2,2'-azobis(2,4-dimethyl valeronitrile, Wako, USA) and solvents (Labscan) were of HPLC or analytical grade. Synthesis products were characterised by ^{1}H-NMR (Varian XL-300 MHz) using tetramethylsilane as external reference, fast atom bombardment (FAB) mass spectrometry (Jeol SX-102, Xe as incident particle, +ve ion mode), TLC, HPLC (as detailed below) and optical rotation (AA-1000, Optical Activity Ltd, England) with samples in methanol solution (c in g ml^{-1}). Monomers were purified prior to use[14] to remove inhibitors.

Dipeptide syntheses

Dipeptides were synthesised by the coupling of the appropriate *N*-acetyl tryptophan and phenylalanine methyl ester enantiomers[15] in the presence of *N,N'*-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole and the products purified by preparative thin layer chromatography [Merck 5717 plates (chloroform/methanol, 95:5)] followed by recrystallisation from dry ethanol. *N-Ac-L-Phe-L-Trp-OMe*, **1**, 83%, mp 62-64 °C. TLC Rf 0.55, $[\alpha]_D^{20} = +11$ (c = 0.010). Found, M+1$^+$ 408.1942, $C_{23}H_{26}N_3O_4$ requires, 408.1960. ^{1}H-NMR [$CDCl_3$] δ 1.79 (s, 3H), $C\underline{H}_3CO$; 3.03 (t, 7.7 Hz, 2H), Phe β; 3.24 (d, J 5.4 Hz, 2H), Trp β; 3.65 (s, 3H), CH_3O; 4.65 (dd, J 4.6, 13.8 Hz, 1H), Phe α; 4.83 (dt, J 5.4, 7.7 Hz, 1H), Trp α; 5.97 (br s, 1H), Trp NH; 6.31 (br s, 1H), Phe NH; 6.90 (d, J 2.5 Hz, 1H), Trp ArH; 7.04 (ddd, J 1.0, 8.1, 1H); 7.13-7.35 (m, 8H), 8 x ArH; 8.18 (br s, 1H), Trp indole NH. *N-Ac-D-Phe-D-Trp-OMe*, **2**, 84%, mp 62-64 °C. TLC Rf 0.55, $[\alpha]_D^{20} = -10$ (c = 0.010). HR-FAB-MS and ^{1}H-NMR spectra were identical in detail to those described for **1**. *N-Ac-D-Phe-L-Trp-OMe*, **3**, 82%, mp 189-191 °C. TLC Rf 0.47, $[\alpha]_D^{20} = -54$ (c = 0.010). *m/z* 407 (M$^+$, 17%). ^{1}H-NMR [$CDCl_3$ + 10% $(CD_3)_2SO$] δ 1.68 (s, 3H), CH_2CO; 2.58-2.68 (m, 2H), 1 x Phe β and 1 x Trp β; 2.85 (dd, J 6.0, 13.7 Hz, 1H), Phe β: 2.99 (d, J 6.3 Hz, 1H), Trp β; 3.47 (s, 3H), OCH_3; 4.49 (ddd, J 1.8, 6.3, 6.6 Hz, 1H), Phe α; 4.57 (ddd, J 1.5, 5.9, 6.3 Hz, 1H), Trp α; 6.74 (d, J 2.3 Hz, 1H), 1 x ArH; 6.83-6.96 (m, 6H), 7 x ArH; 7.16 (d, J 7.8 Hz, 1H), 1 x ArH; 7.25-7.27 (m, 1H), 1 x ArH. *N-Ac-L-Phe-D-Trp-OMe* **4** 80%, mp 189-190 °C. TLC Rf 0.47, $[\alpha]_D^{20} = +54$ (c = 0.010). HR-FAB-MS and ^{1}H-NMR spectra were identical in detail to those described for **3**.

Polymer preparation

A mixture of *N*-Ac-L-Phe-L-Trp-OMe (**1**) (830 mg, 2.03 mmoles), methacrylic acid (**5**) (876 mg, 10.1 mmoles) and ethylene glycol dimethacrylate (**6**) (12.05 g, 60.8 mmoles) were dissolved in chloroform (redistilled. 16 mL) in a screwcap borosilicate glass reaction vial. The mixture was treated with 2,2'-azobis(2,4-dimethyl valeronitrile) (125 mg) then sonicated (3 min) to ensure total dissolution. After cooling to 0 °C, the reaction mixture was sparged with dry nitrogen gas (7 min) then sealed and incubated at 45 °C for 15 hours. The resultant bulk polymer was ground in a mechanical mortar (Retsch, Germany) and wet sieved (water and ethanol) through a 25 µm mesh filter. The material of ≤ 25 µm was sedimented (4 x 30 min) in acetone (400 mL). The MIP was packed (acetone, 300 bar) into stainless steel HPLC columns (200 x 4.6 mm) and washed with methanol/acetic acid (9:1) for 12 h at 1.0 mL min^{-1}.

Polymer analysis

HPLC measurements were conducted on a system comprised of a Waters 501 pump and 484 variable wavelength detector coupled to a Shimadzu C-R3A Chromatopac integrator-recorder. Analyses were run (isocratic) with flow rates of 1.0 mL min^{-1} and detection at 260 or 280 nm. Capacity factors (k') were determined from: $k' = (t-t_0)/t_0$, where t is the retention time of a given species and t_0 that of the void (determined by injection of acetone). Enantio-separation factors (α) were calculated from the relationship: $\alpha = k'_1/k'_2$, where k'_1 and k'_2 are the capacity factors of two individual species, 1 and 2, where 1 is the most retained species. Resolution factors (*f/g*) were determined according to Meyer[16]. Polymer surface areas were determined by single point surface area measurement using a Micromeritics Flowsorb II 2400 instrument

(30% N_2 in He), samples were degassed at 150 °C for 3h prior to determination. Polymer combustion analysis data (Mikro Kemi; Uppsala, Sweden) Found: N, ≤ 0.02 %. Solvent frontal analysis[9] was performed with flow rates of 0.2 mL min^{-1}.

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