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Insights into the role of the hydrogen bond and hydrophobic effect on recognition in molecularly imprinted polymer synthetic peptide receptor mimics

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Abstract

Peptide antibody combining site mimics prepared by the molecular imprinting of N-Ac–L-Phe–L-Trp–OMe were used as highly efficient "tailor-made" chiral stationary phases and for the study of non-ionic non-covalent interaction-based recognition. The effect of water on recognition and the role of the hydrogen bonding and the hydrophobic effect on ligand selectivity are discussed.

1. Introduction

Molecular imprinting [1–3] provides a valuable tool for the modelling of the recognition processes observed in nature [4]. Non-covalent molecularly imprinted polymers (MIPs), in particular, have found application in areas as diverse as artificial antibody combining site mimics [5], artificial enzymes [6–9], synthesis mediators [10,11] and sensor components [12]. The most exploited area of application, however, is in tailor-made chiral chromatographic stationary phase development [3,13,14,15], where the high selectivity of these materials for a *predetermined* ligand makes them versatile systems for custom chromatographic method development.

The preparation of non-covalent molecularly imprinted polymers may be summarized as follows (Fig. 1). For a given imprint or template molecule, a functional monomer (or monomers) is selected with chemical functionality complementary to that of the imprint species. The complementarily interacting functionalities form predictable solution structures [16], the nature of which is captured through polymerization in the presence of a cross-linking monomer. After extraction of the imprint species, recognition sites of complementary steric and functional topography to the imprint molecule remain. Subsequent incubation of a mixture of the imprint molecule and related chemical species results in the selective rebinding of the imprint structure. The imprinting process thus engenders a cognitive capacity to the polymer. It is this selective recognition capability which is utilized in molecularly imprinted polymer-based chiral stationary phases (CSPs). MIP CSPs are distinguished from other CSPs by their predictable order of elution, whereby the application of a racemate of the imprint species always results in longer retention times for the imprinted enantiomer. Individual enantiomer dissociation constants reflect the differing affinities for MIP recognition sites.

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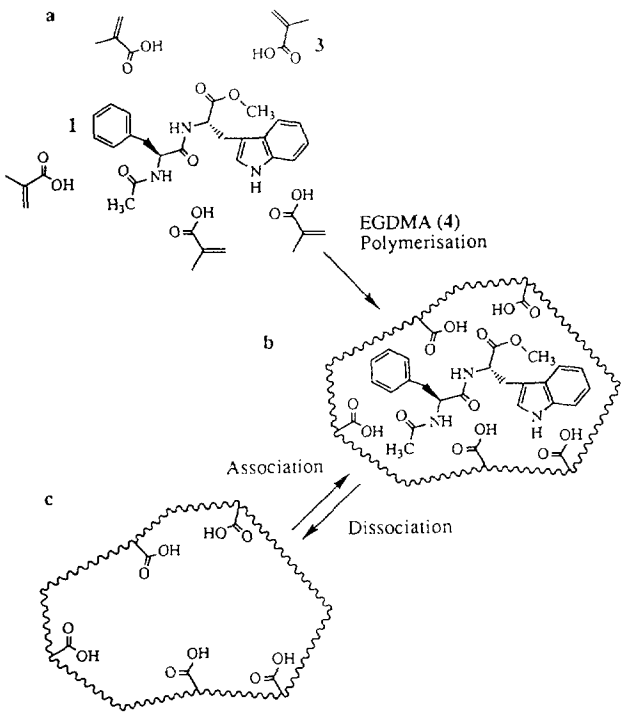

Fig. 1. Idealized molecular imprinting of N-Ac-L-Phe-L-Trp-OMe (**1**) in a methacrylic acid (**3**)–ethylene glycol dimethacrylate (**4**) copolymer. (a) Hydrogen-bonding interactions between functional monomers and the imprint species define solution adducts; (b) topographical relationship present in the solution adduct is captured by polymerization; (c) imprint species extraction yields a recognition site of complementary shape and functional topography.

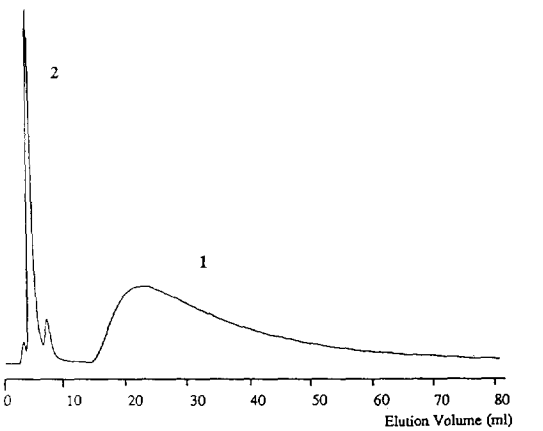

Fig. 2. Chromatogram of the resolution of a racemate of Ac–Phe–Trp–OMe (i.e., **1** and **2**), using chloroform (1% AcOH) as eluent (10 μg injected). The attenuation was increased ten-fold after 13 min to provide a more clear view of the peak of **1**.

A current goal in the development of new MIPs is the study of recognition in aqueous media. MIP receptor mimics selective for the dipeptide N-Ac-L-Phe-L-Trp-OMe (**1**) have recently [13] been shown to be capable of resolving racemic (Fig. 2) and diastereomeric mixtures of the imprint structure and racemates of a range of structurally related compounds. The unprecedented separations achieved with this system make it well suited to the study of recognition in solvent systems less favourable to the formation of hydrogen bonds, the strongest driving force for recognition in non-polar media [3]. Previous studies, with less selective MIPs based on methacrylic acid (MAA) (**3**)–ethylene glycol dimethacrylate (EGDMA) (**4**) copolymers, have been limited to the use of organic eluents. We report here the effect of aqueous–organic mixtures on recognition, which permits conclusions to be drawn regarding the role of the hydrogen bond and hydrophobic effect on recognition.

2. Experimental

2.1. General methods

Reagents and chemicals (Sigma, St. Louis, MO, USA), initiator [2,2′-azobis(2,4-dimethylvaleronitrile), Wako, USA] and solvents (Labscan, Malmö, Sweden) were of HPLC or analytical-reagent grade. Synthesis products were characterized 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, positive-ion mode), TLC, HPLC (as detailed below) and optical rotation (AA-1000; Optical Activity, Cambridge, UK) with samples in methanol solution (c in g ml^{-1}). Monomers were purified prior to use [17] to remove inhibitors. Combustion analyses were performed by Mikrokemi (Uppsala, Sweden).

2.2. Dipeptide syntheses

Dipeptides were synthesized [18] by the coupling of the appropriate enantiomers of N-acetylphenylalanine and tryptophan methyl ester in the presence of N,N′-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The products were purified by preparative TLC [Merck 5717 plates, chloroform–methanol (95:5) as eluent] followed by recrystallization from dry ethanol. Spectroscopic characterization of these materials has been described previously [13]. N-Ac–L-Phe–L-Trp–OMe (**1**), $[\alpha]_D^{20} = +11°$ ($c = 0.010$); N-Ac–D-Phe–D-Trp–OMe (**2**), $[\alpha]_D^{20} = -10°$ ($c = 0.010$).

2.3. Polymer preparation

Imprinted and blank polymers were prepared and characterized as described previously [13]. A mixture of N-Ac–L-Phe–L-Trp–OMe (**1**) (2.00 mmol), methacrylic acid (**4**) (5 equiv.) and ethylene glycol dimethacrylate (**5**) (30 equiv.) were dissolved in chloroform (redistilled, 16 ml) in a screw-capped borosilicate glass reaction vial. The mixture was treated with 2,2′-azobis(2,4-dimethylvaleronitile) (125 mg) then sonicated (3 min) to ensure total dissolution of reactants. After cooling to 0°C, the reaction mixture was sparged with dry nitrogen (7 min), then sealed and incubated at 45°C for 18 h. The resultant bulk polymer was ground in a Retsch Model RM0 mechanical mortar (Haan, Germany) and wet sieved (water and ethanol) through a 25-μm mesh filter. The material of ≤ 25 μm was sedimented (4×30 min) in acetone (400 ml). The MIP was packed (acetone, 300 bar) into stainless-steel HPLC columns (150×4.6 mm I.D.) and washed with methanol–acetic acid (7:3) for 18 h at 1.0 ml min^{-1}.

2.4. Polymer analysis

HPLC measurements were conducted on an LKB system consisting of two Model 2150 pumps, a Model 2152 gradient controller and a Model 2151 variable-wavelength detector coupled to a Waters WISP Model 710B autoinjector and interfaced with a Shimadzu C-R3A Chromatopac integrator–recorder. Analyses were run at flow-rates of 1.0 ml min^{-1} with detection at 260 nm. Capacity factors (k') were determined from $k' = (v - v_0)/v_0$, where v is the retention volume of a given species and v_0 is the void volume (determined by injection of acetone). Effective enantioseparation 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.

3. Results

The *anti*-N-Ac–L-Phe–L-Trp–OMe MIP was prepared [13] utilizing MAA (**3**) as the functional monomer, which is capable of acting as both a hydrogen bond donor and acceptor with the imprint species (Fig. 3). The bulk polymer was processed to render it suitable for use as a CSP for HPLC, packed into HPLC columns, then washed exhaustively under acidic conditions to remove residual imprint species. Combustion analysis data indicated a nitrogen content, in washed and dried polymer samples, consistent with extraction of $\geq 95\%$ of the print species. It was assumed that any residual print molecule was inaccessible to the bulk solvent.

The MIP's capacity for resolution of the imprint species (**1**) from its stereoisomer, N-Ac–D-Phe–D-Trp–OMe (**2**) was examined (Table 1) using pure acetonitrile and acetonitrile–water mixtures containing acetic acid. The relative insolubility of the dipeptides in aqueous mixtures

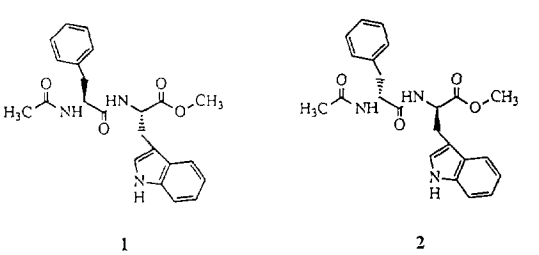

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Fig. 3. Structure of the peptides separated.

Table 1
N-Ac–Phe–Trp–OMe enantiomeric recognition by the *anti*-N-Ac–L-Phe–L-Trp–OMe (**1**) MIP

Water (%, v/v)	k'_{DD} (**2**)	k'_{LL} (**1**)	α'
0	2.6	8.0	3.30
2	1.5	2.0	1.33
5	1.6	1.8	1.13
8	1.1	1.2	1.02

Chromatographic capacity factors (k') and *effective* separation factors (α') for the separation of LL-(**1**) and DD-(**2**) N-Ac–Phe–Trp–OMe (15 μg of dipeptide were injected in each run) using acetonitrile-based buffer systems.

precluded more than an 8% water content being used in the eluent. The acidic modifier was incorporated in the eluent mixtures to maintain polymer carboxylic acid residues in their protonated form and so preclude ion–dipole interactions between the polymer carboxylate moieties and the analytes. This was manifested as a sharpening of peak shape (reduction in tailing) corresponding to a reduction in non-specific binding modes. Analysis using a "blank" polymer, one prepared in the absence of the imprint species, afforded no evidence of stereo-differentiation in any instance. Solvent frontal analysis [19] (Table 2) demonstrates the enantiomer-dependent dissociation constants (k_{diss}) for **1** and **2** and provides estimates of the number of binding site populations for each enantiomer.

4. Discussion

Highly enantioselective separations have recently been reported utilising an *anti*-N-Ac–L-Phe–L-Trp–OMe MIP [13]. In organic media (chloroform), the selective recognition of the imprint species, the LL-dipeptide (**1**) relative to its DD-enantiomer (**2**), affords a clear baseline separation [Fig. 2, α-value 17.8 (note: 200 × 4.6 mm I.D. column and flow-rate 1.0 ml min^{-1}]. This is reflected in the different dissociation constants and binding site populations for the two enantiomers as determined by solvent frontal analysis (Table 2).

Table 2
Solvent frontal zone analysis of the *anti*-N-Ac–L-Trp–OMe MIP

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

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.

Effective separation factors, α', calculated from the injection of individual enantiomers, rather than racemates, can be of use in evaluating stereoselective recognition in systems where small reproducible differences in retention times are present [13]. To investigate the role of water on recognition, an aqueous compatible organic solvent, acetonitrile, was selected. The α' values determined for **1** and **2** in acetonitrile were substantially lower than have previously been observed when using less polar eluents such as chloroform [13] (Table 1). On introduction of increasing amounts of water into the eluent mixture, a decrease in retention times was observed for both enantiomers, although most significantly for the imprint species. Enantiomeric differentiation, however, was still observed, on comparison of capacity factors and the calculation of *effective* separation factors.

The significance of hydrogen bonding has been enunciated in earlier discussions on recognition in MIP systems [3,4,16] and has been shown to be the major factor for recognition in MIPs prepared in non-polar solvents. The presence of water significantly lowers the effectiveness of the hydrogen bonding between ligand and receptor due to competition for hydrogen bond donor and acceptor sites. In accord with these facts, the effect of the presence of water was more pronounced in the case of the selective rebinding of the imprint species (**1**) than of its optical antipode (**2**). This suggests that the contribution of hydrophobic interactions for recognition in this system is mainly non-specific in character. This arises, we propose, from the fact that hydrophobic interactions [20], as distinct from Van der Waals complementarity [21], are not as significant in the defining of imprint recognition sites during the prearrangement phase of MIP prepa-

ration [4] in non-polar media. Thus, the hydrophobic binding of these ligands to the MIP is mainly non-specific, and applied similarly to both stereoisomers.

That **2** is partially recognized under conditions favourable to hydrogen bonding 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 imprint species chromatographic response, the "primary recognition peak", hence conclusions reflect upon the nature of the "average site" rather than in terms of a uniform site population. Again, the dissociation constants determined for the imprint species (**1**) and its enantiomer (**2**) by solvent frontal zone analysis (Table 2) 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. Further, this technique provided a means of determining the binding site populations for the individual enantiomers. The difference between these two values provides a measure of the number of sites specific for **1**, *i.e.*, 2.1 μmol per gram (dry mass) of polymer under the conditions examined.

Molecular imprinting offers a means of constructing recognition sites of predetermined selectivity and is thus an extremely useful tool for the construction of "tailor-made" separation media and for the study of molecular recognition phenomena. The levels of enantioselectivity demonstrated by this *anti*-N-Ac-L-Phe-L-Trp-OMe MIP have previously only been observed for systems utilizing ionic interaction-facilitated recognition [3]. The dipeptide receptors have been used to investigate the influence of water on recognition in methacrylic acid–ethylene glycol dimethacrylate MIPs. Finally, MIP tailor-made recognition systems are now capable of racemate resolution on a par with the best commercially available CSPs and possess remarkable chemical and mechanical stability, as demonstrated by their repeated use, in many hundreds of runs, over several months.

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