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Molecular recognition in synthetic polymers: preparation of chiral stationary phases by molecular imprinting of amino acid amides

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ABSTRACT

Methacrylate-based molecular imprints were prepared using a number of L-amino acid aromatic amide derivatives as print molecules. Methacrylic acid was used as a functional monomer such that the acid function of the monomer interacts ionically with the amine function and via hydrogen bonding with the amide function of the print molecule. Bulk polymers were prepared and were ground and sieved to particles <25 μm, packed into high-performance liquid chromatographic (HPLC) columns and used for enantiomeric separations in the HPLC mode. The polymers were shown to exhibit efficient enantiomeric resolution of a racemic mixture of the amino acid amide used as the print molecule and in many instances were also able to resolve the enantiomers of amino acid amides other than the print molecule, depending on the substituents on the amine and amide functionalities. Allowing an increased number of monomers to interact with the print molecule, *i.e.*, by introducing an additional amide function or a pyridyl ring to the print molecule, led to an improved separation in most instances, although increased band broadening was observed, especially when isocratic elutions were performed. With all polymers, acetic acid gradient elution improved the peak shape, leading to increased resolution and shorter analysis times. The implications of these findings with respect to the mechanism of recognition and the ability to predict the enantiomeric resolution of substances on molecularly imprinted polymers are discussed.

INTRODUCTION

Molecular imprinting in synthetic polymers is a new and potentially very interesting technique for preparing specialized separation media for chromatography,

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especially for enantiomeric separations. Polymerization is allowed to occur in the presence of a template, the print molecule, which is subsequently removed from the rigid polymer, thereby producing sites within the polymer with affinity for the original print molecule, the so called "memory" effect. Polymerizable monomers are chosen to allow specific and definable interactions with the print molecule. Such interactions are subsequently responsible for the recognition of the substrate by the polymer. The interactions can be either non-covalent forces such as ionic and hydrogen bonding[1,2] or the formation of reversible covalent bonds, such as ketals[3], boronic esters[4,5] and Schiff's bases[6,7].

We have previously described the preparation of molecular imprints of amino acid derivatives[2] and in particular we have been interested in the application of the technique of molecular imprinting to the optical resolution of such compounds. Previously described polymers showed very high enantio selectivity and were sufficiently rigid to be used in the high-performance liquid chromatography (HPLC) mode[8]. In addition, the enantiomers of structurally related compounds could be resolved on a polymer of predefined specificity[9]. A solution NMR study gave evidence for the formation of complexes between a print molecule (L-phenylalanine anilide) and functional monomers (methacrylic acid) defined by ionic and hydrogen bonds[10]. It was argued that these complexes were preserved during the polymerization process, resulting in an arrangement of methacrylic acid residues in the polymer responsible for subsequent recognition of molecules. From the ability of the polymer to resolve the enantiomers of an array of structural analogues of the print molecule, it was possible to draw some conclusions about the mechanism of recognition[9]. It was proposed that the substrate was initially bound to the polymer via "ion pairing" between the primary amine of the substrate and a carboxylic acid residue on the polymer. Additional interactions between the polymer and substrate, such as hydrogen bonding between the amide on the substrate and carboxylic acid residues in the polymer, were then "induced".

We have extended these earlier investigations and describe here the molecular imprinting of eight amino acid derivatives based on the same structural elements previously shown to be important for enantiomeric resolution in this system. All print molecules used in this study were amino acid amide derivatives. The aromatic amide was either an anilide or a β-naphthylamide and various substituents were coupled to the amino nitrogen. The versatility of the resulting polymers to resolve the enantiomers of amino acid amide derivatives was analysed in the HPLC mode. The aim was to correlate the chromatographic properties of the polymers with the structures of the print molecules. The effect of increasing the number of interactions between the print molecule and monomers during polymerization on the ability of the resulting polymers to separate enantiomers and the applications of these polymers as chiral stationary phases in column chromatography are discussed.

EXPERIMENTAL

Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDMA) were obtained from Aldrich Chemie (Steinheim, F.R.G.), 2,2′-azobis(2-methylpropionitrile) (AIBN) from Janssen Chemica (Beerse, Belgium), L-leucine-β-naphthylamide (LeuβNA) and L-phenylalanine-β-naphthylamide (PheβNA) from Sigma (St. Louis,

MO, U.S.A.) and D-leucine-β-naphthylamide from Bachem (Bubendorf, Switzerland). D- and L-phenylalanine anilides (PheAn), D- and L-proline anilides (ProAn) and D- and L-phenylalanylglycine anilides (PheGlyAn) were all synthesized by a procedure similar to that described previously for L-phenylalanine anilide[8]. D- and L-N,N-dimethylphenylalanine anilides (Me_2PheAn) were prepared by reductive methylation of phenylalanine anilide analogously to a literature method[11]. D- and L-N-pyridoxylphenylalanine anilides (PLPheAn) were prepared essentially as described elsewhere[12]. The syntheses of D- and L-N-(pyridylmethyl)phenylalanine anilides (PyMePheAn) were developed in our laboratory and will be described in detail elsewhere[13]. Briefly, phenylalanine anilide was reacted with pyridine-4-carbaldehyde in acetonitrile to produce the imine, which was then reduced by sodium cyanoborohydride.

All solvents used were of the highest available grade. HPLC analyses were performed with an LKB (Bromma, Sweden) system consisting of a Model 2152 HPLC controller two Model 2150 HPLC pumps and a Model 2151 variable-wavelength monitor. Fourier transform IR (FT-IR) analyses were performed with a Nicolet 20 SXC instrument.

Polymer preparation

Polymers were prepared as described previously[8,14] using EDMA as cross-linker and MAA as the functional monomer. The compositions of the polymerization mixtures are shown in Table I. The molar ratio of cross-linker to functional monomer to print molecule was 30:6:1.5 in entries A–C, E and F; in entries D, G and H a molar ratio of 30:6:1 was used. MAA, EDMA, initiator (AIBN), chloroform and the appropriate amount of crystalline print molecule were weighed into 50-ml borosilicate glass ampoules (Wheaton Scientific, Melvill, NJ, U.S.A.). The mixtures were cooled on ice, degassed under vacuum in a sonicating bath and sparged with nitrogen for 5 min. The ampoules were then sealed with Parafilm and placed under a UV source (366 nm) at 4°C overnight (16 h). The bulk polymers were ground in a mechanical

TABLE I

POLYMER PREPARATIONS

All polymers were prepared using EDMA (52.4 mmol) as cross-linking monomer, chloroform (16 ml) as solvent and AIBN (0.76 mmol) as the initiator at 0°C as described previously.

Polymer	*Print molecule*			*MAA (mmol)*	*Ratio MAA:pm*[a]	*Inner surface area (m^2/g)*[b]
	Abbreviation	*mg*	*mmol*			
A	L-PheβNA	760	2.62	10.48	4	3.8
B	L-LeuβNA	671	2.62	10.48	4	3.3
C	L-PheAn	629	2.62	10.48	4	6.2
D	L-PheGlyAn	520	1.75	10.48	6	5.6
E	L-ProAn	498	2.62	10.48	4	4.2
F	L-Me_2PheAn	702	2.62	10.48	4	2.8
G	L-PyMePheAn	579	1.75	10.48	6	3.5
H	L-PLPheAn	684	1.75	10.48	6	3.6

[a] Ratio MAA:pm refers to the molar ratio of functional monomer (MAA) to print molecule (pm).
[b] Inner surface area was determined by nitrogen adsorption measurements.

mortar (Retsch, Haan, F.R.G.) and wet sieved (water) through a 25-μm sieve (Retsch). The fraction that passed through the sieve was collected and the remainder was re-ground. This procedure was repeated until all polymer particles passed through the sieve. The fines were removed from the preparation by repeated settling in acetonitrile and the particles were finally dried under vacuum.

High-performance liquid chromatography

Particles were suspended in chloroform by sonication and packed into 200 mm × 4.5 mm I.D. stainless-steel columns using acetonitrile as solvent with an air-driven fluid pump (Haskel Engineering Supply, Burbank, CA, U.S.A.) at 300 bar. The columns were then washed on-line with methanol–acetic acid (9:1, v/v) until a stable baseline was obtained. HPLC analyses were performed under either isocratic or gradient elution conditions. Isocratic elutions were performed with acetonitrile containing the appropriate percentage of acetic acid (5–12.5%, v/v) at a flow-rate of 1 ml/min and with detection at 250 nm. Gradient elutions were performed using a linear gradient of 0–30% (v/v) acetic acid in acetonitrile over 30 min at a flow-rate of 1 ml/min. Samples consisted of a mixture of 5 μg of each of the L- and D-enantiomers of a given compound, prepared in acetonitrile, and injected in a total volume of 20 μl.

Enantiomeric resolution was confirmed by separate injections of each of the enantiomers. The void volume of the columns were determined by injection of glacial acetic acid. Capacity factors (k'), separation factors (α) and plate numbers (N) were calculated using standard chromatographic theory[15]. The resolution (R_s) was calculated according to Wulff *et al.*[4].

RESULTS AND DISCUSSION

Polymer preparations

A recently developed polymerization procedure, based on photolytic initiation, was used in this study as the chromatographic properties of the resulting polymers have previously been shown to be superior to polymers prepared by thermal initation[8]. All polymerizations were performed under equivalent conditions to ensure that the physical properties of the polymers were as equivalent as possible. In addition, the composition of the polymerization mixture was kept constant throughout all experiments, with the exception of added print molecule. The molar ratio of cross-linker (EDMA) to functional monomer (MAA) was 5:1. The optimum molar ratio of MAA to print molecule was previously shown to be about 4:1[9,14] and this ratio was used here, except in entries D, G and H (Table I). To allow for the possibility of an additional interaction point to the print molecules L-PheGlyAn, L-PyMePheAn and L-PLPheAn, the ratio of functional monomer to print molecule was increased to 6:1 by decreasing the amount of print molecule added to the polymerization mixtures of these polymers (Table I). As the formation of complexes between print molecule and functional monomers in the prepolymerization mixture is an equilibrium process, the introduction of additional interaction points on the print molecule must be accompanied by a corresponding increase in the amount of functional monomer. This was achieved by keeping the number of moles of interacting sites constant, rather than the number of moles of print molecule, by decreasing the amount of print molecule added to the polymerization mixture. In this way the ratio of functional monomer (MAA) to

the number of "potential" interaction sites was constant in all polymerization mixtures.

As a routine measure of the equivalence of physical properties between polymer preparations, the specific surface areas were determined. As can be seen from the data in Table I, all polymer preparations had comparable surface areas. In this context it should be noted that a "reference" polymer, prepared in the absence of print molecule, possessed different physical properties. The specific surface area was 13 m^2/g of polymer for the reference polymer compared with 3–6 m^2/g for imprinted polymers A–H (Table I) and the pore volume of the reference polymer was 0.08 cm^3/g compared with 0.04–0.05 cm^3/g for polymers C and F (not shown).

The polymer particles were packed into stainless-steel columns (200 × 4.5 mm I.D.) and washed on-line with 10% (v/v) acetic acid in methanol to remove print molecules from the polymer. The amount of irreversibly incorporated print molecules, determined by FT-IR difference spectra between polymer C (Table I) and a reference polymer (prepared in the absence of print molecule), was calculated to be less than 1% of the total amount of print molecules added, as no IR bands originating from PheAn could be detected (not shown).

Chromatography

Polymers were evaluated in the HPLC mode using either isocratic or gradient elution schemes. When using isocratic elution conditions the composition of the eluent was optimized for each stationary phase by changing the concentration of acetic acid. In order to compare chromatographic data from all stationary phases, the eluent was chosen to give a capacity factor, k', for the D-form of the print molecule of *ca.* 1. Eight polymers were prepared (see Table I) using print molecules containing the same basic structural elements; all were aromatic amides of amino acids, as shown in Fig. 1. Racemic mixtures of all amino acid amides were analysed on all eight columns and the results are presented as separation factors, α, in Table II, and a few representative chromatograms are depicted in Fig. 2.

The broad elution peaks obtained in the present system, particularly for the more strongly retained L-forms of the substrates, may be due to several factors, including the size distribution and shape irregularity of the polymer particles. In addition, broad elution peaks may be due to differences in the number and complementarity of interactions between the print molecule and functional monomers, prior to and during polymerization, giving rise to a wide range of non-equivalent sites in the polymer. A third explanation for peak broadening may be that the kinetics of release are slow such that $K_{ass} \gg K_{diss}$, as discussed previously[9]. The observed peak broadening is probably due to a combination of all of these factors. Peak broadening was most pronounced on polymers D, G and H (Table I), imprinted against compounds L-PheGlyAn, L-PyMePheAn and L-PLPheAn, respectively, with an increased number of interaction points with the functional monomer. Despite the increase in separation factor on polymers D and G (see below), the resolution was not improved and therefore lower than expected for these columns; $R_s = 0.5$ (polymer D) and $R_s = 1.1$ (polymer G) compared with $R_s = 0.7$ (polymer B) and $R_s = 1.0$ (polymer C). This was probably due to band-broadening effects, particularly as all the columns used in this study resulted in symmetrical void peaks with plate numbers between 500 to 650 using glacial acetic acid (not shown).

TABLE II

SEPARATION FACTORS[a] FOR THE DIFFERENT AMINO ACID DERIVATIVES ON POLYMERS A–H (TABLE I)

A mixture of 5 μg of each of the enantiomers of the amino acid derivatives was injected onto the column in a total volume of 20 μl of acetonitrile and eluted at room temperature under isocratic conditions using the appropriate percentage of acetic acid in acetonitrile. Detection was at 250 nm. Plate numbers, calculated for a non-retained void marker (acetic acid), were approximately the same for all columns, between 500 and 650. The separation factors obtained for print molecule on respective columns are underlined.

Polymer	*Print molecule*[b]	*Acetic acid concentration (%)*[c]	*Substrate*						
			LeuβNA	*PheAn*	*PheGlyAn*	*ProAn*	*Me_2PheAn*	*PyMePheAn*	*PLPheAn*
A	L-PheβNA[d]	12.5	(2.4	5.5	3.0	1.0	1.0	1.0	1.0)[d]
B	L-LeuβNA	10	3.8	4.8	2.2	1.0	1.0	1.0	1.0
C	L-PheAn	10	2.6	4.1	2.1	1.2	1.0	1.0	1.0
D	L-PheGlyAn	7.5	1.0	3.0	5.1	1.0	1.0	1.0	1.0
E	L-ProAn	7.5	2.8	2.5	1.0	4.5	1.0	1.0	1.0
F	L-Me_2PheAn	7.5	1.0	1.0	1.0	1.0	3.7	1.0	1.0
G	L-PyMePheAn	5	1.0	1.0	1.0	1.0	1.0	8.4	1.0
H	L-PLPheAn	5	1.0	1.0	1.0	1.0	1.0	1.6	2.7

[a] Separation factor, α, is the ratio k'_L/k'_D.
[b] Structures are shown in Fig. 1.
[c] Acetic acid concentration (%) refers to the percentage of acetic acid in acetonitrile used during chromatography.
[d] Only the L-form of PheβNA was applied as substrate and separation factor, α, could not be calculated for this compound.

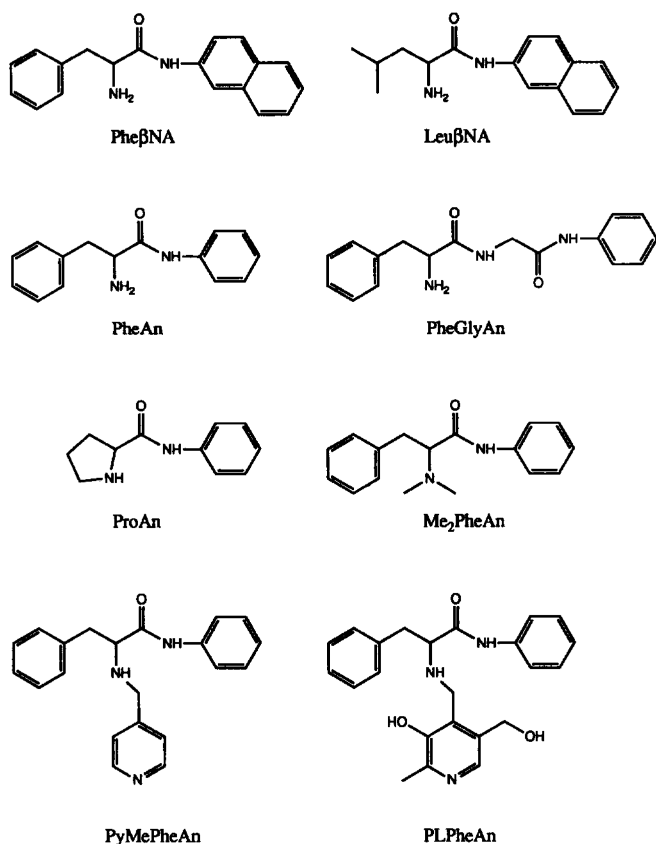

Fig. 1. Structures of the compounds used as print molecules. Abbreviations: PheβNA, phenylalanine-β-naphthylamide (polymer A); LeuβNA, leucine-β-naphthylamide (polymer B); PheAn, phenylalanine anilide (polymer C); PheGlyAn, phenylalanylglycine anilide (polymer D); ProAn, proline anilide (polymer E); Me_2PheAn, N,N-dimethylphenylalanine anilide (polymer F); PyMePheAn, N-pyridylmethylphenylalanine anilide (polymer G); PLPheAn, N-pyridoxylphenylalanine anilide (polymer H).

As acetic acid is a competing ligand, it follows that by increasing the acetic acid concentration in the eluent, the equilibrium would be shifted towards dissociation, or elution of the substrate, and the higher the K_a the higher is the concentration of acetic acid required to effect elution. It was considered, therefore, that gradient elution [0–30% (v/v) acetic acid in acetonitrile] would both improve the peak shape and give some information on the relative affinities of the substrates for the various polymers, as reflected in the percentage of acetic acid required for elution (see Table III). Fig. 3 shows the elution profiles for the enantiomers of PLPheAn on polymer H using both isocratic and gradient elution. In this instance, the resolution showed a modest increase from 0.4 to 0.5 and the analysis time decreased from 30 to 20 min when gradient elution was used.

Specificity of recognition

In a previous study we showed that it was possible to resolve the enantiomers of

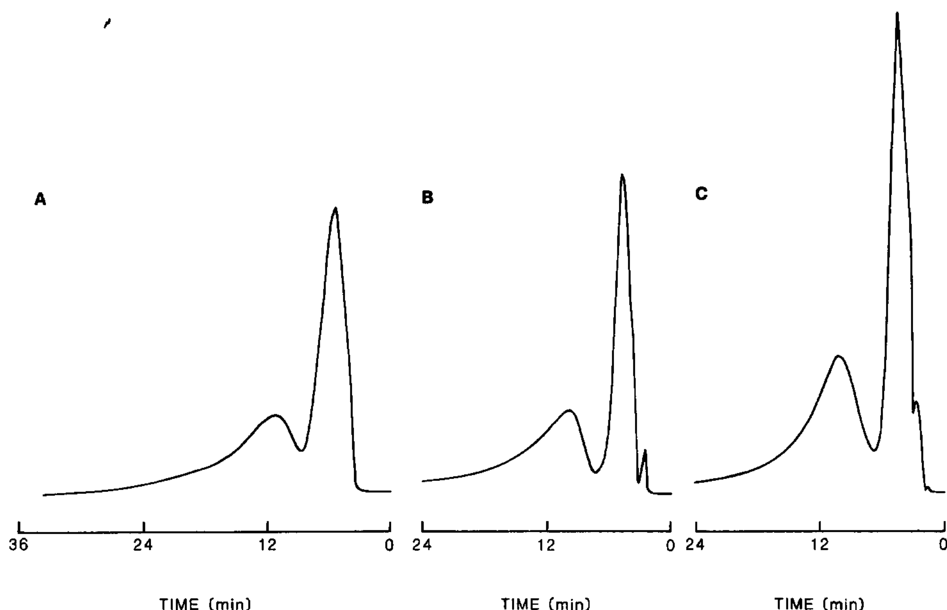

Fig. 2. Enantiomeric resolution of amino acid amides. Particles of <25 μm were packed into 200 × 4.5 mm I.D. columns. Analyses were performed isocratically using (A) 10% (v/v) or (B and C) 7.5% (v/v) acetic acid in acetonitrile as the eluent at 1 ml/min at room temperature. Detection was at 250 nm. In all experiments, a mixture of 5 μg of each of the enantiomers of the compound was analysed. Analyses shown: (A) D,L-PheAn on polymer C (prepared against L-PheAn, Table I); (B) D,L-Me$_2$PheAn on polymer F (prepared against L-Me$_2$PheAn, Table I); and (C) D,L-ProAn on polymer E (prepared against L-ProAn, Table I).

TABLE III

SEPARATION OF THE ENANTIOMERS OF THE PRINT MOLECULE BY GRADIENT ELUTION ON POLYMERS A–H (TABLE I)

A mixture of 5 μg of each of the enantiomers of the corresponding amino acid derivative was injected onto the column in a total volume of 20 μl of acetonitrile and eluted with acetonitrile–acetic acid using a linear gradient of 0–30% acetic acid in 30 min. Detection was at 250 nm.

Polymer	*Print molecule*	*Elution*[a]	
		L	*D*
A	L-PheβNA	15.7	–
B	L-LeuβNA	11.0	6.6
C	L-PheAn	14.5	10.7
D	L-PheGlyAn	9.5	8.4
E	L-ProAn	10.1	7.3
F	L-Me$_2$PheAn	10.5	7.1
G	L-PyMePheAn	10.1	6.9
H	L-PLPheAn	7.9	6.3

[a] Percentage of acetic acid at which the compound eluted.

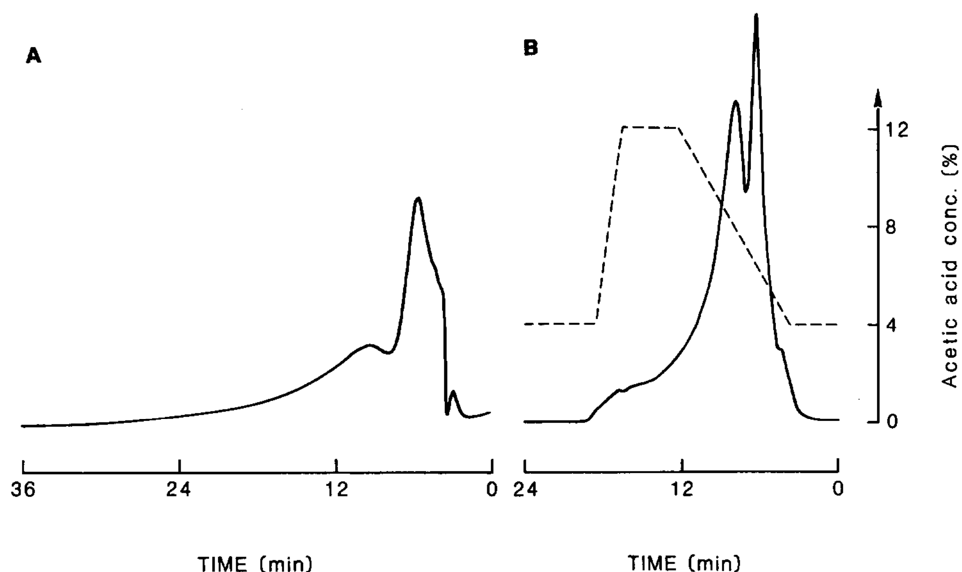

Fig. 3. Resolution of the enantiomers of the print molecule on polymer H (prepared against L-PLPheAn, Table I). Particles of <25 μm were packed into a 200 × 4.5 mm I.D. column. (A) Analysis performed at room temperature with acetonitrile–acetic acid (19:1, v/v) as the eluent at 1 ml/min; (B) analysis with the following gradient of acetic acid in acetonitrile at 1 ml/min: 0–4 min, 4%; 4–12 min, 4–12%; 12–16 min, 12%; 16–18 min, 12–4%. Detection was at (A) 250 nm and (B) 290 nm. Samples consisted of 5 μg of each of the enantiomers of PLPheAn in a total volume of 20 μl of acetonitrile. The resolution (R_s), calculated as in ref. 4, was *ca.* (A) 0.4 and (B) 0.5. Asymmetry factors (A_s) at 10% of the peak height were calculated to be (A) 3.7 and (B) 2.7 for the L-peak and (A) 1.5 and (B) 0.9 for the D-peak.

a number of compounds on a polymer molecularly imprinted against L-PheAn[9] and that an amine and an amide, in the correct geometry around the chiral carbon, were necessary for resolution. In this work we extended previous investigations by preparing molecular imprints against eight aromatic amides of amino acids. The structural differences between the print molecules were small; the amide moiety was either an anilide or β-naphthylamide, and the amine moiety was either primary, secondary or tertiary (see Fig. 1). All compounds had the same arrangement of amine and amide around the chiral carbon, previously shown to be essential for enantiomeric resolution on a polymer prepared against L-PheAn[9]. This ensures, as far as possible, that the types of interactions involved in the recognition process are the same on all polymers, *viz.*, ionic bonding to the amine and hydrogen bonding to the amide. The introduction of additional interactions, such as hydrogen bonding in PheGlyAn and ionic bonding in PyMePheAn (see below), should not alter the type of interactions but only increase their number. The recognition sites formed after polymerization would, in principle therefore be very similar in structure and any differences would be indicative of changes in the three-dimensional arrangement, spatial or distal, of carboxylic acid residues, the number of carboxylic acids or the shape (volume) of the sites. The elution data for the various amino acid amides should then reflect differences in the conformation, configuration and number of interactions introduced into the recognition sites by a particular print molecule.

Racemic mixtures of all amino acid amides were analysed on all eight stationary phases prepared and the data are presented in Table II as separation factors, α. It is important to note that enantiomeric resolution was observed for all stationary phases, at least for the print molecule. In this context, a polymer imprinted against a racemic mixture of D,L-PyMePheAn, did not display any enantiomeric resolution (not shown).

In almost all instances the best separation was achieved when the enantiomers of the print molecule were applied to the column (Table II). Although the polymers were able to resolve the enantiomers of compounds other than the print molecule, the separation factor, α, was less than that for the racemate of the print molecule. Unexpectedly, on many polymer preparations only the enantiomers of a few amino acid amides, in some instances only of the print molecule, were resolved, at least using the present elution schemes.

The first group of polymers to be discussed are polymers A, B, C and D, prepared against L-PheβNA, L-LeuβNA, L-PheAn and L-PheGlyAn, respectively. On all four polymers enantiomeric resolution was observed when a racemic mixture of each of the compounds LeuβNA, PheAn and PheGlyAn was applied to the columns, with the exception of LeuβNA on polymer D. These observations extend and support the hypothesis that an amine moiety and an amide moiety, in the correct geometry around the chiral carbon, are the major criteria for enantiomeric recognition on molecularly imprinted polymers[9]. When applying compounds containing a substituted amine, as with ProAn, Me_2PheAn, PyMePheAn and PLPheAn, no enantiomeric resolution could be detected on polymers A–D. Therefore, these polymers have the ability to distinguish, at least to some extent, primary from substituted amines.

Polymers E, F, G and H prepared against the "substituted" amines L-ProAn, L-Me_2PheAn, L-PyMePheAn and L-PLPheAn, respectively, showed little tendency to resolve the enantiomers of compounds other than the original print molecule, with a few exceptions (see Table II). It must be stressed, however, that the possibility exists that a polymer prepared against L-Me_2PheAn, for example, may be able to resolve the enantiomers of a number of other N,N-dimethylated amino acid amides, analogous to polymers A–D and previous observations[9].

The mutual inability of polymers prepared against substituted and unsubstituted amines to resolve the enantiomers of the other type of amine can not be accounted for by differences in ionic interactions, as the pK_a values for the corresponding acids of primary, secondary and tertiary amines are generally in the same range of 10–11. The substituents on the amino groups would therefore only cause the participants in the ionic bond to become more distanced from each other, but as coloumbic interactions are long range on the molecular level the ionic bond is not expected to be weakened to any great extent. This is supported by the observation that regardless of the number of substituents on the amine moiety, all amino acid amides were bound approximately equally strongly to the polymers in the present solvent system, in that all D-forms and L-forms of the non-resolved substances eluted at approximately the same position on any given polymer (data not shown). Perhaps the most striking examples of the selectivity for unsubstituted and substituted amines are polymers C and F, imprinted against L-PheAn and L-Me_2PheAn, respectively (see Table II), where the addition or removal of two methyl groups to the amine of the print molecule resulted in total loss of enantiomeric resolution. This effect cannot be simply

explained by differences in ionic bonding to carboxylic acid residues on the polymer but may be related to the shape of the molecules[16] and hence the shape of the binding sites on the polymers and/or additional interactions such as hydrophobic interactions or changes in hydrogen bonding abilities. It is also unlikely that the bulkiness of the two methyl groups would be a major factor responsible for the observed effect.

By increasing the number of possible interactions between polymer and substrate it was expected that the resolution of the enantiomers would improve. The introduction of an additional point of interaction into the print molecule should allow coordination of more functional monomers during polymerization and subsequent incorporation of additional carboxylic acid residues into the recognition sites. This concept was investigated on polymers D, G and H, imprinted against L-PheGlyAn, L-PyMePheAn and L-PLPheAn, respectively. PheGlyAn has a peptide bond in the molecule (Fig. 1) and therefore additional hydrogen bonding potential. PyMePheAn has a pyridine ring coupled to the amine (Fig. 1), which should result in an additional ionic bond to the pyridine nitrogen, albeit weak as the pK_a of the pyridine ring is *ca.* 5. PLPheAn was included in the investigation because of its complexity, being a more functionalized molecule than the other compounds investigated. It is similar in structure to PyMePheAn but contains an additional hydroxyl group and a hydroxymethyl group on the pyridine ring (Fig. 1). In each of the three compounds, these "additional" points of interaction are situated at a distance from the chiral carbon, but if this interaction is participating in the chiral recognition event an improved enantiomeric separation would be expected. Polymer D, prepared against L-PheGlyAn, produced a significantly higher separation factor for the enantiomers of the print molecule compared with polymers B and C, prepared against "similar" print molecules, L-LeuβNA and L-PheAn (Table II). Polymer G, imprinted against L-PyMePheAn, resolved the enantiomers of the print molecule very efficiently (Table II). It is in fact one of the highest α-values ever obtained in our laboratories and among the highest ever reported in the literature in the field of molecular imprinting[2]. Peak broadening of the L-enantiomer was considerable but the D-enantiomer eluted as a narrow, symmetrical peak (not shown). Analysis of a racemic mixture of D- and L-PLPheAn on polymer H produced a lower separation factor than expected.

Although the second amide moiety in PheGlyAn and the pyridyl nitrogen in PyMePheAn are situated at a distance from the chiral carbon in these molecules, the additional interactions had a positive effect on the separations. In general, the results indicate that additional points of interaction between the print molecule and functional monomers during polymerization gives rise to polymers possessing improved separation abilities, exemplified by polymers D and G, imprinted against L-PheGlyAn and L-PyMePheAn, respectively. However, this is not universally applicable, as shown by polymer H, imprinted against L-PLPheAn.

CONCLUSIONS

It is clear from the results presented here and in previous publications[8–10,12,14] that some form of chiral information is introduced into the polymer during polymerization in the presence of a template, the print molecule. This information is preserved during work-up of the polymer and removal of print molecule, resulting in the "memory effect". The polymeric material can then be used as a chiral stationary

phase in column chromatography. It is unlikely that the observed enantiomeric separations are due to irreversible incorporation of chiral molecules as the template is almost quantitatively removed after polymerization, as determined in the present study by FT-IR measurements. The enantiomeric separations observed are more likely due to specific interactions between the substrate and chiral recognition sites within the polymer. The fact that the best separation is almost always recorded for the enantiomers of the print molecule supports this hypothesis. Although it is extremely difficult to "map" the actual structure of these sites, several assumptions may be made. It seems clear that one or several carboxylic acid residues from functional monomers are incorporated into the sites[10]. These acid functionalities are responsible for the initial binding of substrate to the polymer via ionic interaction with the amino function(s) on the substrate[9]. The forces giving rise to enantiomeric separations are more difficult to study.

An indirect approach to obtaining information about the enantiomeric separation event is to investigate different racemates and record which compounds are resolved, as described here. It was shown previously that an amine and an amide in the correct geometry around the chiral carbon are crucial for a substrate to be resolved on a polymer prepared against L-phenylalanine anilide[9]. The side-chain on the amino acid derivative is of little importance for enantiomeric resolution. Here we have presented data showing that the moieties important for binding to the polymer are of extreme importance for chiral resolution, *e.g.*, the type of amine in the amine–carboxylic acid interaction. It should be stressed that binding and chiral resolution are two distinct events. Increasing the number of interactions between the substrate and the polymer results in higher separation factors but the unfavourable kinetics on such polymers lead to peak broadening. For practical reasons it is neither necessary nor advantageous to increase the number of interactions in order to achieve better separations, at least for small organic molecules. Although the type of amine determines if a given amino acid amide is resolved on a particular polymer, it is recognized that all amino acid amides are bound equally strongly to each polymer. We conclude that the initial binding of the substrate to the polymers occurs via ionic bonding between the amine on the substrate and carboxylic acid residues on the polymer. The second step in the recognition process is the formation of other interactions, such as hydrogen bonds and hydrophobic forces, and it is these "secondary" interactions which give rise to the enantiomeric separations observed.

Molecular imprinting is an interesting and simple technique for preparing chiral stationary phases for enantiomeric separations. The relatively high separation factors achieved in the present system and in previous studies[8–10,14] are promising for future developments of polymers with practical applications in column chromatography. The polymers are rigid and stable enough to be used at high pressures and are also resistant to most of the commonly used solvents[9]. The major shortcoming of these polymer preparations in column chromatography is the sometimes pronounced broadening of peaks. Gradient elution schemes improve the peak shape, increase the resolution and shorten analysis times. However, further improvements in the chromatographic applications of these polymers are desirable and may include the preparation of beaded molecular imprints and a thorough investigation of the kinetics of association and dissociation.

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