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Enantiomeric resolution on molecularly imprinted polymers prepared with only non-covalent and non-ionic interactions

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ABSTRACT

Molecular imprints were prepared utilizing only weak bonds between the print molecule and functional monomers; the bonding forces used in the imprinting process were only those weaker than covalent and ionic bonds. Methacrylate-based molecular imprints were prepared using a number of chiral compounds, including N-protected amino acid derivatives, as print molecules. Methacrylic acid was used as the functional monomer because the acid function of the monomer forms hydrogen bonds with a variety of polar functionalities, such as carboxylic acids, carbamates, heteroatoms and carboxylic esters, of the print molecule. Bulk polymers were prepared, ground and sieved to particles of size <25 μm, packed into high-performance liquid chromatographic (HPLC) columns and used for enantiomeric separations in the HPLC mode. The polymers were shown to effect efficient enantiomeric resolution of a racemate of the print molecule in addition to substrate selectivity for the print molecule in a mixture of substrates with very similar structures. For example, the enantiomers of Cbz-aspartic acid and Cbz-glutamic acid (Cbz = carbobenzoxy) were resolved with separation factors of 1.9 and 2.5, respectively, on polymers with molecular imprints of the L-form of the respective compounds. In addition, these polymers, prepared against Cbz-L-aspartic acid and Cbz-L-glutamic acid, respectively, had the ability to bind selectively the print molecule from a mixture of both racemates, although the two compounds differ only by one methylene group. The results presented represent a substantial widening of the scope of molecular imprinting in that it may now be possible to prepare molecular imprints against a very large number of compounds.

INTRODUCTION

The advances made recently in the field of molecular imprinting have been considerable, especially in the preparation of separation media for enantiomeric resolutions. The preparation of molecular imprints of amino acid derivatives and of other compounds by the non-covalent pre-arrangement approach has been described

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in a recent review[1]. Molecular imprints can be prepared against amino acid esters[2] and amino acid amides[3–7] using methacrylic acid as the functional monomer; this monomer interacts ionically with the amine of the print molecule, and its use is referred to below as the "ionic system". Such polymers can be used as stationary phases in the high-performance liquid chromatographic (HPLC) mode for enantiomeric separations of amino acid derivatives. A different approach to the preparation of molecular imprints involves the formation of reversible covalent bonds between monomers and a print molecule (for a review, see ref. 8), referred to below as the "covalent system". The reversible covalent bonds may be ketals[9], Schiff bases[10,11] or boronic esters[12,13]. However, the number of suitable reversible covalent bonds available for molecular imprinting is very limited; an adduct between the print molecule and suitable monomer(s) must be synthesized and the print molecule must be removed quantitatively from the polymer after the polymerization. Likewise, the number of compounds suitable for molecular imprinting utilizing ionic bonds is limited. In this paper this issue is addressed and we show that the limitations described can be overcome.

The role of hydrogen bonding in molecular recognition has been the focus of much attention recently[14]. It has long been known that carboxylic acids form hydrogen bonds with a great variety of polar functionalities, including carbamates, amides, carboxylic acids and carboxylic esters, and that the hydrogen bonds connecting one carboxylic acid with another are very strong[15]. By condensing Kemp triacid with various diamines, compounds were prepared that contained carboxylic acid functions arranged in a convergent molecular cleft for the selective binding of amines and neutral molecules[16]. Synthetic macrocyclic receptors in which the host–guest interactions are claimed to be based mainly on hydrogen bonds have also been reported[17,18]. These findings were applied to molecular imprinting, and in this paper we report for the first time the preparation of molecular imprints utilizing only bonds weaker than covalent and ionic between the print molecule and functional monomers. The interactions, during both the polymerization and the subsequent recognition event, are based solely on hydrogen bonds and other weak forces, such as hydrophobic interactions and dipole–dipole interactions. The resulting polymers were analysed for their ability to separate the enantiomers of print molecules and also for their ability to separate the original print molecule from a mixture of compounds similar in structure.

EXPERIMENTAL

Ethylene glycol dimethacrylate (EDMA) was obtained from Polysciences (Warrington, PA, U.S.A.) and methacrylic acid (MAA), 2,2′-azobis(2-methylpropionitrile) (AIBN) and *d*- and *l*-mandelic acids from Janssen Chimica (Beerse, Belgium). D- and L-Boc-tryptophans, D- and L-Boc-phenylalanines, D- and L-Boc-proline-N-hydroxysuccinimide esters and D- and L-Cbz-tryptophans were obtained from Nova Biochem (Läufelfingen, Switzerland) and D- and L-Cbz-aspartic acids and D- and L-Cbz-glutamic acids from Bachem (Bubendorf, Switzerland) (Boc = *tert.*-butoxycarbonyl; Cbz = carbobenzoxy, benzyloxycarbonyl). D- and L-Cbz-tryptophan methyl esters were prepared essentially as described in the literature[19]. Solvents were of either analytical-reagent or HPLC 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.

Polymer preparation

Polymers were prepared according to a standard method described previously[4,5] using methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EDMA) as cross-linker. The molar ratio of cross-linker to functional monomer to print molecule was 20:4:1, except for polymers prepared against Cbz-L-aspartic acid and Cbz-L-glutamic acid, where a molar ratio of 20:4:0.5 was used. MAA (10.48 mmol), EDMA (52.4 mmol), AIBN (0.76 mmol), chloroform (16 ml) and the appropriate amount of print molecule (2.62 or 1.31 mmol) 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 water 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 mortar (Retsch, Haan, F.R.G.) and sieved through a 25-μm sieve (Retsch). The fines were then removed by repeated sedimentation in acetonitrile and the particles were finally dried under vacuum.

High-performance liquid chromatography

Particles were suspended in chloroform–acetonitrile (17:3, v/v) by sonication and packed into 200 mm × 4.5 mm I.D. stainless-steel columns with acetonitrile as solvent at 300 bar using an air-driven fluid pump (Haskel Engineering Supply, Burbank, CA, U.S.A.). The columns were then washed on-line with methanol–acetic acid (9:1, v/v) until a stable baseline was obtained. The print molecule was almost quantitatively removed from the polymer by this treatment, as judged by Fourier transform IR measurements[7]. HPLC analyses were performed isocratically with the solvent compositions and flow-rates indicated for each polymer preparation in Tables I and II. Detection was at 250 nm. A mixture of 5 μg of each of the enantiomers of a given compound, prepared in the mobile phases, was injected for analysis in a total volume of 20 μl. Enantiomeric resolution was confirmed by separate injections of each of the enantiomers. The void volumes of the columns were determined by injection of acetone and chloroform. Capacity factors (k'), separation factors (α) and plate numbers (N) were calculated using standard chromatographic theory[20]. The resolution (R_s) was calculated according to Wulff *et al.*[12].

RESULTS AND DISCUSSION

The chromatographic performance of columns containing molecularly imprinted polymers has often been poor, as indicated by the broad peaks obtained. This has been attributed to mass transfer limitations, including both diffusion resistance and slow rates of binding and relase of the substrate. Previously, molecularly imprinted columns were run at elevated temperatures and low flow-rates[2,3,12]. A light-induced polymerization procedure was developed that could be performed at low temperatures (0°C)[4,5]. This method is easy to perform and the resulting polymer preparations display a significantly increased enantioselectivity in comparison with those prepared by previous methods. The resulting increase in resolving capacity allowed the preparation of columns that could be run at acceptable flow-rates at ambient temperature[4]. In addition, the peak shapes could be improved even further by applying gradient elution schemes[7]. The polymer preparations described here were all prepared using this improved polymerization procedure.

In the initial phase of this study we prepared molecular imprints against N-protected amino acids (see Fig. 1). The carboxylic acid function of the functional monomers was expected to form strong hydrogen bonds with the carboxylic acid function of the print molecules. Additionally, the methacrylic acid monomers may form hydrogen bonds with the carbamate function of the print molecules. The nitrogen in the pyrrole ring of the tryptophan derivatives may also form hydrogen bonds with the methacrylic acid monomers[15]. The polymer preparations were evaluated for their ability to resolve the enantiomers of print molecules in the chromatographic mode. In some instances the enantiomeric resolution of other substrates, similar in structure to the print molecule, was also analysed. Typical chromatograms of racemates of print molecules on polymers with molecular imprints against carboxylic acid derivatives are depicted in Fig. 2.

The results of the chromatographic evaluations of polymers with molecular imprints against the dicarboxylic acid derivatives Cbz-L-aspartic acid and Cbz-L-

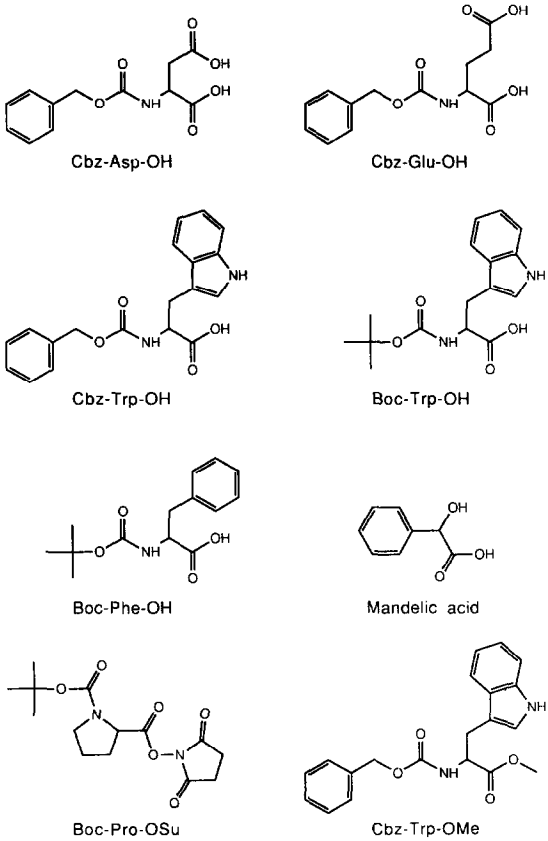

Fig. 1. Structures of the compounds used as print molecules. Abbreviations: Cbz-Asp-OH = N-benzyloxycarbonylaspartic acid; Cbz-Glu-OH = N-benzyloxycarbonylglutamic acid; Cbz-Trp-OH = N-benzyloxycarbonyltryptophan; Boc-Trp-OH = N-*tert.*-butoxycarbonyltryptophan; Boc-Phe-OH = N-*tert.*-butoxycarbonylphenylalanine; Boc-Pro-OSu = N-*tert.*-butoxycarbonylproline N-hydroxysuccinimide ester; Cbz-Trp-OMe = N-*tert.*-benzyloxycarbonyltryptophan methyl ester.

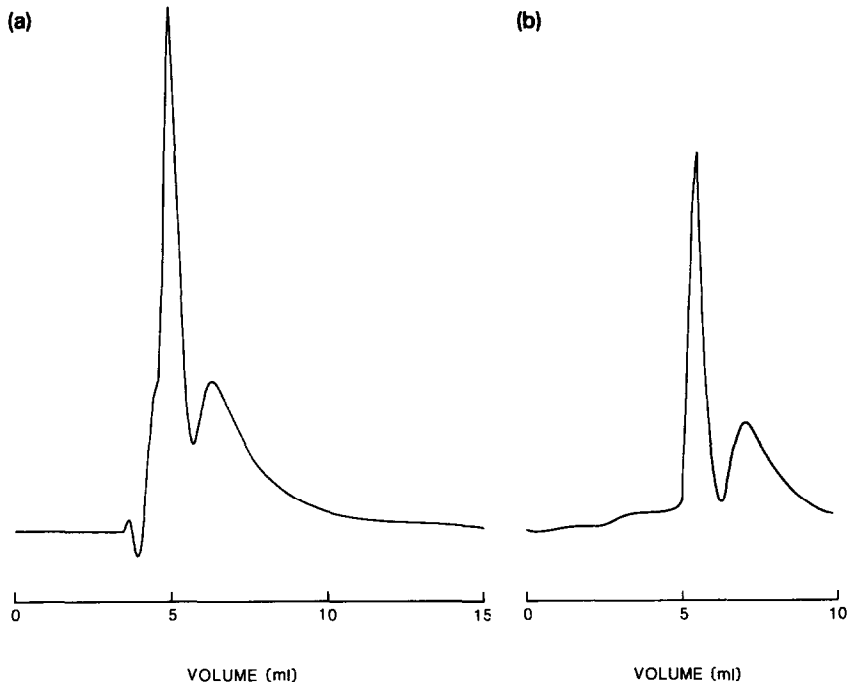

Fig. 2. Enantiomeric resolution of print molecule on polymers prepared with molecular imprints against Boc-L-tryptophan (Table II) and Boc-L-phenylalanine (Table II). Particles (<25 μm) were packed into 200 × 4.5 mm I.D. columns. Analyses were performed isocratically using (a) acetonitrile–chloroform–acetic acid (90:9.5:0.5, v/v/v) and (b) 0.25% (v/v) acetic acid in chloroform 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. Plate numbers calculated for a non-retained, non-interacting void marker (acetone) were $N = 826$ for the column used in A and $N = 880$ for the column used in B. The analyses shown are (a) Boc-D,L-tryptophan on a polymer prepared against Boc-L-tryptophan and (b) Boc-D,L-phenylalanine on a polymer prepared against Boc-L-phenylalanine.

glutamic acid are presented in Table I. The enantiomers of the print molecule were resolved very efficiently on the respective polymer preparations. An interesting observation was that whereas analysis of a racemate of the "wrong" dicarboxylic acid gave no enantiomeric resolution with the present elution system (see Table I), injection of the pure enantiomers produced distinct peak maxima with separation factors in the range 1.05–1.08.

Analyses of a mixture of the two racemates, Cbz-aspartic acid and Cbz-glutamic acid, on these polymer preparations are shown in Fig. 3. The print molecule was the most retained compound on the respective polymer preparations, showing that efficient substrate selectivity between compounds very similar in structure was possible. For polymers molecularly imprinted against amino acid amides in the ionic system, the separation of the amino acid amide derivatives on a column with pre-defined specificity is highly dependent on the substitution of the amine moiety[7], whereas the side groups are of little importance[6]. In this respect, the extreme substrate selectivity of the dicarboxylic acid polymers is unexpected (Cbz-aspartic acid and Cbz-glutamic acid differ only by one methylene group, see Fig. 1).

TABLE I

ENANTIOMERIC RESOLUTION ON POLYMERS MOLECULARLY IMPRINTED WITH CBZ-L-ASPARTIC ACID AND CBZ-L-GLUTAMIC ACID

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 mobile phase. The elution was performed isocratically at room temperature using acetonitrile–chloroform–acetic acid (60:39.5:0.5, v/v/v) at a flow-rate of 1 ml/min (entries 1A and 2A) or 0.1 ml/min (entries 1B and 2B).

Entry	*Print molecule*	*N (acetone)*	*Cbz-aspartic acid*						*Cbz-glutamic acid*					
			k'_D	k'_L	N_D	N_L	α	R_s	k'_D	k'_L	N_D	N_L	α	R_s
1A	Cbz-L-Asp-OH	345	0.58	1.11	267	87	1.91	0.9	0.62	0.62	348[a]	281[a]	1.0	n.d.[b]
1B	Cbz-L-Asp-OH	820	0.58	1.25	500	142	2.16	1.7	0.61	0.61	564[a]	491[a]	1.0	n.d.
2A	Cbz-L-Glu-OH	538	0.49	0.49	458[a]	373[a]	1.0	n.d.	0.74	1.81	407	91	2.45	1.4
2B	Cbz-L-Glu-OH	995	0.51	0.51	n.d.	n.d.	1.0	n.d.	0.75	1.90	950	156	2.53	2.9

[a] Calculated on separate injections of the enantiomers, as injection of a racemate produced only a broad, partially resolved peak.

[b] n.d. = Not determined.

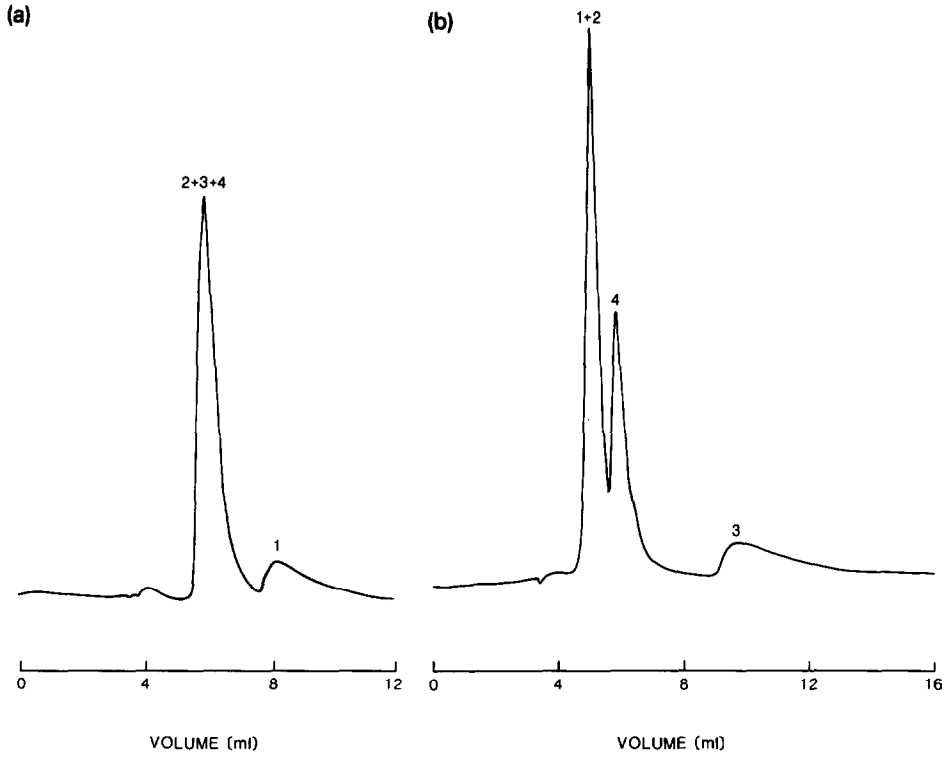

Fig. 3. Separation of (1) Cbz-L-aspartic acid, (2) Cbz-D-aspartic acid, (3) Cbz-L-glutamic acid and (4) Cbz-D-glutamic acid on polymers prepared against (a) Cbz-L-aspartic acid and (b) Cbz-L-glutamic acid. Particles (< 25 μm) were packed into 200 × 4.5 mm I.D. columns. Analyses were performed isocratically using acetonitrile–chloroform–acetic acid (60:39.5:0.5, v/v/v) as the eluent at 0.1 ml/min at room temperature. Detection was at 250 nm. In both expriments, a mixture of 10 μg of each of the racemates of Cbz-aspartic acid and Cbz-glutamic acid was analysed.

The results of the molecular imprinting of the tryptophan derivatives Boc-L-tryptophan (see Fig. 2) and Cbz-L-tryptophan are presented in Table II. The enantiomers of the respective print molecules were resolved on both polymer preparations. In addition, the enantiomers of Cbz-tryptophan were resolved ($\alpha = 1.2$) on the polymer preparation made against Boc-L-tryptophan and the enantiomers of Boc-tryptophan were resolved ($\alpha = 1.3$) on the polymer preparation made against Cbz-L-tryptophan. This is analogous to previous findings with the ionic system[6,7]. Enantiomeric resolution of a racemate of the print molecule was also possible on polymers prepared with molecular imprints against other carboxylic acid derivatives, such as Boc-L-phenylalanine (see Fig. 2 and Table II) and *l*(−)-mandelic acid (see Table II).

After the successful preparation of polymers with molecular imprints of carboxylic acid derivatives, we examined print molecules with even weaker hydrogen bonding interactions with the functional monomers. Polymers were prepared against Boc-L-proline hydroxysuccinimide ester (Boc-L-Pro-OSu) and Cbz-L-tryptophan methyl ester (see Fig. 1) and analysed in the chromatographic mode. On both polymer

TABLE II

ENANTIOMERIC RESOLUTION OF THE ENANTIOMERS OF PRINT MOLECULES ON MOLECULARLY IMPRINTED POLYMERS

The eluents used were acetonitrile–chloroform–acetic acid (90:9.5:0.5, v/v/v) at a flow-rate of 1 ml/min (entries 1 and 2A) or 0.1 ml/min (entry 2B); 0.25% (v/v) acetic acid in chloroform at a flow-rate of 1 ml/min (entry 3); 0.5% (v/v) acetic acid in chloroform at a flow-rate of 0.5 ml/min (entry 4); chloroform–heptane (1:1, v/v) at a flow-rate of 1 ml/min (entry 5A) or 0.1 ml/min (entry 5B); and chloroform–heptane (3:1, v/v) at a flow-rate of 1 ml/min (entry 6A) or 0.1 ml/min (entry 6B). Other conditions as in Table I.

Entry	*Print molecule*	N_{void}[a]	k'_D	k'_L	N_D	N_L	α	R_s
1	Boc-L-Trp-OH	826	0.43	0.83	449	68	1.90	0.8
2A	Cbz-L-Trp-OH	449	0.56	0.94	147	25	1.67	0.1
2B	Cbz-L-Trp-OH	573	0.56	1.11	184	31	1.98	0.6
3	Boc-L-Phe-OH	825	0.68	1.21	783	143	1.77	1.4
4	*l*(−)-Mandelic acid	825	2.00	2.87	383	131	1.43	1.1
5A	Boc-L-Pro-OSu	771	1.10[b]	1.24[b]	435[b]	377[b]	1.1[b]	–
5B	Boc-L-Pro-OSu	825	1.10	1.38	537	403	1.25	0.8
6A	Cbz-L-Trp-OMe	463	1.41	1.80	345	78	1.28	0.2
6B	Cbz-L-Trp-OMe	771	1.41	2.05	776	213	1.46	1.5

[a] The void markers were acetone (entries 1–4) and chloroform (entries 5–6).

[b] Calculated on separate injections of the enantiomers, as injection of a racemate produced only a broad, partially resolved peak.

preparations the enantiomers of the print molecule were efficiently resolved (see Table II). The weaker hydrogen bonds between these substrates and the carboxylic acid functions of the polymers were recognized by the necessity to use less polar eluents to achieve suitable retardations in the successful chromatographic separations.

The mass transfer limitations in the HPLC separation are obvious, as shown by the observation that the enantiomeric separation increased and the resolution improved considerably when the flow-rate was decreased from 1 to 0.1 ml/min (Tables I and II). The observed increase in α is a result of the increase in k' for the L-form (the enantiomer used as the print molecule) at the low flow-rate, whereas the elution of the D-form was unchanged (see Tables I and II). As expected for columns packed with particles of the size used in this study (25 μm)[20], the plate numbers, N, for the D- and L-peaks and the void marker peak increased when the flow-rate was decreased, resulting in better resolution (see Tables I and II). A flow-rate of 0.1 ml/min is too low from a practical point of view; a flow-rate of 1 ml/min is routinely used in the studies of molecularly imprinted polymers. However, conducting parallel analyses at low flow-rates is justified in order to emphasize the potential for development of these chiral separation media. It is important to note that the chromatographic efficiency of these polymer preparations compared well with that reported for microcrystalline triacetylcellulose[21], a widely used chiral separation medium (Table I).

The results presented here clearly show that it is possible to prepare molecular imprints in synthetic polymers even if the coordination of functional monomers with the print molecule is not held together by strong covalent or ionic bonds. In the chromatographic separations it is a clear advantage if only weak forces between the substrate and the solid support operate, because the rates of binding and release are increased and the amount of competing ligand needed in the eluent is decreased. In this

context it should be mentioned that in the covalent system a catalyst was added to the eluent to speed up the binding reaction[12], and in the ionic system a high percentage of competing ligand (acetic acid) was added to the eluent[7]). The lower separation factors in the present system (α = 1.1–2.5 compared with 4–8 in the ionic system[7] are compensated for by the better chromatographic performance, resulting in resolutions which are as good as, and in some instances superior to, those with the previous ionic system.

CONCLUSIONS

Interest in molecular imprinting has grown in the last few years, as a means of producing stationary phases for column chromatography[1], as a model system for studying the recognition of a substrate by a macromolecule[1,8,22] and as a tool eventually to obtain enzyme-like catalysts[23–25]. Two basically similar approaches for the preparation of molecular imprints have been utilized in the past, involving either ionic[1] or covalent[8] bonds between the print molecule and the functional monomer(s). In this study we have shown that molecular imprinting is not limited to a small number of exclusive compounds which can form reversible covalent or ionic bonds with the functional monomer(s).

Enantiomeric resolution of print molecules was observed for all the stationary phases prepared; in the best cases the resolving capability was very good. The enantiomeric resolutions of substrates other than the print molecule were always less than that for the print molecule if they were resolved at all, at least with the eluent systems used. Substrate selectivity between compounds with very similar structures, as exemplified by the pair Cbz-aspartic acid and Cbz-glutamic acid, is efficient in the present system. The results presented here represent a substantial widening of the scope of molecular imprinting in that it may now be possible to prepare molecular imprints against a large number of substances.

The polymers used in this present study could be used in column chromatography both as a chiral stationary phase (pre-determined enantioselectivity) and as a normal phase (pre-determined substrate selectivity). The major shortcoming of these polymer preparations in column chromatography at present appears to be the sometimes pronounced broadening of peaks; however, as shown here, the potential exists for further improvements in column performance. Studies of the kinetics of binding and release are warranted and optimizations may include the preparation of beaded particles of defined size and shape.

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