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RECENT ADVANCES IN THE PREPARATION AND USE OF MOLECULARLY IMPRINTED POLYMERS FOR ENANTIOMERIC RESOLUTION OF AMINO ACID DERIVATIVES

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SUMMARY

Acrylate-based molecular imprints were prepared, using L-phenylalanine anilide as the print molecule and methacrylic acid as the functional monomer, which is believed to interact both ionically and through hydrogen bonding with the print molecule. Several aspects of the polymer preparation were investigated, including the solvent of polymerization, the initiation system and the effect of the molar ratio of functional monomer to print molecule on the ability of the polymers to separate the enantiomers of the print molecule. Polymers were analysed by high-performance liquid chromatography where the effect of particle size and eluent composition were investigated for enantiomeric resolution of the print molecule. The ability of the polymers to separate racemic mixtures of amide derivatives of amino acids other than phenylalanine anilide (print molecule) was also investigated. It was thus shown that efficient enantiomeric resolution of amide derivatives of amino acids including the anilides, *p*-nitroanilides, *β*-naphthylamides and amides of amino acids, ranging from alanine to tryptophan, was possible on a single polymer imprinted with L-phenylalanine anilide. Such separations were highly specific and dependent on the presence of both the print molecule and functional monomer in the polymerization mixture. The mechanism of recognition was shown to involve ionic bonding to the primary amine and hydrogen bonding to the amide function of the substrate by acid groups of the polymer, "immobilized" in a stereospecific manner. Both interactions were necessary for efficient enantiomeric resolution. Molecular imprints, prepared from L-phenylalanine anilide, were also shown to give efficient enantiomeric resolution of phenylalanine-containing dipeptides and may be useful for preparative-scale separations.

INTRODUCTION

Two basically similar approaches have been followed for the preparation of molecular imprints for a variety of molecules (for a review see ref. 1). The first involves the formation of reversible covalent adducts between the print molecule and functional monomers. Examples of this include the formation of Schiff bases[2,3], boronic esters[4,5] and ketals[6]. The second approach involves the use of non-covalent

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interactions, such as ionic and hydrogen bonding, between the print molecule and functional monomers[7]. In both cases, these interactions are allowed to occur in solution prior to initiation of polymerization and are subsequently responsible for recognition of molecules by the polymer.

In our laboratory, we have used the approach of non-covalent interactions for the preparation of molecular imprints for amino acid derivatives[7–12]. These polymers are subsequently used in high-performance liquid chromatography (HPLC) for enantiomeric resolution of racemic mixtures of amino acid derivatives. In the present paper we present some recent advances in the preparation and use of molecular imprints with amino acid derivatives as the print molecules. A new polymerization procedure, which allows the preparation of polymers at 0°C, is presented. The method of preparation of the polymers is simpler to perform, is less time-consuming and results in polymers exhibiting greater separation abilities than those previously presented. In addition, several factors affecting the performance of such polymers in HPLC for enantiomeric resolution were investigated. Finally, the versatility of molecular imprints to separate racemic mixtures of molecules other than the print molecule has been demonstrated and the results allow some conclusions to be drawn on the mechanism of recognition.

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 Chimica (Beerse, Belgium) and 2,2′-azobis(2,4-dimethylvaleronitrile) (ABDV) from Polysciences (Warrington, PA, U.S.A.). The print molecule, L-phenylalanine anilide, was synthesized as previously described[11]. D-Phenylalanine anilide and D- and L-tyrosine and tryptophan anilides were synthesized in a similar manner. D- and L-phenylalanylglycine ethyl esters were synthesized from glycine ethyl ester and D- and L-Boc-phenylalanine, respectively, using dicyclohexyl carbodiimide and hydroxybenzotriazole as condensing agents[13] in dimethylformamide. The Boc protecting group was then removed by treatment with trifluoroacetic acid in dichloromethane. All other amino acid derivatives were obtained from either Bachem (Bubendorf, Switzerland) or Nova Biochem (Läufelfingen, Switzerland) and were used without further purification. Solvents were of either analytical or HPLC grades. HPLC analyses were performed with a LKB (Bromma, Sweden) system comprising a Model 2152 HPLC controller, two Model 2150 HPLC pumps and a Model 2151 variable-wavelength monitor.

Polymer preparation

Polymers were prepared by either thermal initiation or photolytic initiation with azobisnitriles (AIBN or ABDV)[11]. The composition of the standard polymerization mixture, determined from the experiments described herein, is shown in Table I. The crystalline print molecule (L-phenylalanine anilide), functional monomer (MAA), cross-linking monomer (EDMA), initiator and solvent (most commonly chloroform) were weighed into 50-ml borosilicate glass ampoules (Wheaton Scientific, Melvill, NJ, U.S.A.). Solubilization was achieved by sonication and the mixture was then cooled on ice. The mixture was degassed under vacuum in a sonicating bath

TABLE I

STANDARD POLYMERIZATION MIXTURE

Crystalline print molecule (L-phenylalanine anilide), functional monomer (MAA), cross-linking monomer (EDMA), initiator (AIBN) and solvent (chloroform) were added to 50-ml borosilicate glass ampoules, and complete solubilization was achieved by sonication.

Addition	*mg*	*mmol*	*mol %*[a]
Print molecule	474	1.956	4
MAA	677	7.86	16
EDMA	7790	39.3	80
AIBN	94.5	0.57	–
Solvent		12 ml	

[a] Calculated by neglecting the initiator (AIBN). Molar ratio MAA/print molecule is 4:1.

and sparged with nitrogen for 5 min. Then the ampoules were sealed with Parafilm. For thermal initiation, the ampoules were placed in a water-bath at the temperature of decomposition of the initiator (AIBN, 60°C; ABDV, 40°C). For photoinitiation, the ampoules were irradiated at 366 nm, using a standard laboratory UV source, at 4°C. In all cases, polymerization was allowed to proceed overnight (18 h), after which the ampoules were smashed. The polymer was ground in a mortar and wet-sieved in water to the desired size distribution. Particles which passed through a 25-μm sieve constituted a fraction of <25 μm. In all cases, dust was removed by flotation in acetonitrile, and the particles were finally dried under vacuum.

High-performance liquid chromatography

For particles of 45–63 μm, HPLC columns (100 mm × 5 mm or 200 mm × 5 mm) were dry-packed. Particles <25 μm were slurried by sonication in water–acetonitrile–acetic acid (10:9:1; v/v/v) and packed using the same solvent at 300 bar using an air-driven fluid pump (Haskel Engineering Supply, Burbank, CA, U.S.A.). All columns were then washed on-line with acetonitrile–acetic acid (9:1; v/v) at a flow-rate of 1 ml/min until a stable baseline was obtained. HPLC analyses were performed isocratically with either 19:1 (v/v) or 9:1 (v/v) acetonitrile–acetic acid at a flow-rate of 1 ml/min and detection at 260 nm. Analyses were performed at room temperature. Samples to be analysed were prepared in either acetonitrile or acetic acid, depending on their solubility, and were injected in a total volume of 20 μl. Routinely, a mixture of 25 μg of each of the L- and D-enantiomers of a given compound was injected for analysis. Enantiomeric resolution was confirmed by separate injections of each of the enantiomers. All samples analysed contained 1 μg of a non-interacting, non-excluded void marker, Boc-L-phenylalanine anilide purified as an intermediate in the synthesis of the print molecule, L-phenylalanine anilide[11].

RESULTS

Factors affecting printing efficiency and column performance

Previous descriptions of the preparation of molecular imprints have predominantly involved the use of thermal initiators[1–10]. Most commonly, AIBN has been

used at polymerization temperatures of 60–120°C. We were interested in investigating the effect of the polymerization temperature on the subsequent performance of the polymers for two major reasons. First, it was considered that the interactions between the print molecule and the functional monomers would be stronger at lower temperature, thereby increasing the printing efficiency. Secondly, polymerization at elevated temperatures was considered to be inappropriate for the imprinting of temperature-sensitive molecules.

Initial investigations on the effect of polymerization temperature involved the preparation of polymers at 60°C, using AIBN, and at 40°C using ABDV as the initiators. Analyses of these polymers in HPLC for the separation of the enantiomers of the print molecule (L-phenylalanine anilide) showed that polymerization at 40°C resulted in a small but significant increase in the observed separation. We therefore sought to decrease the temperature of polymerization even further through the use of photosensitizers and photoinitiators. It was subsequently realized that the azobisnitriles undergo photolytic decomposition when irradiated at 366 nm (refs. 14 and 15) and it was demonstrated that molecular imprints could be prepared using this type of initiation at temperatures as low as 0°C[11]. Polymers prepared in this manner resulted in excellent enantiomeric resolution of a racemic mixture of the print molecule (Fig. 1). The results shown in Fig. 1 were obtained with a polymer prepared using photoini-

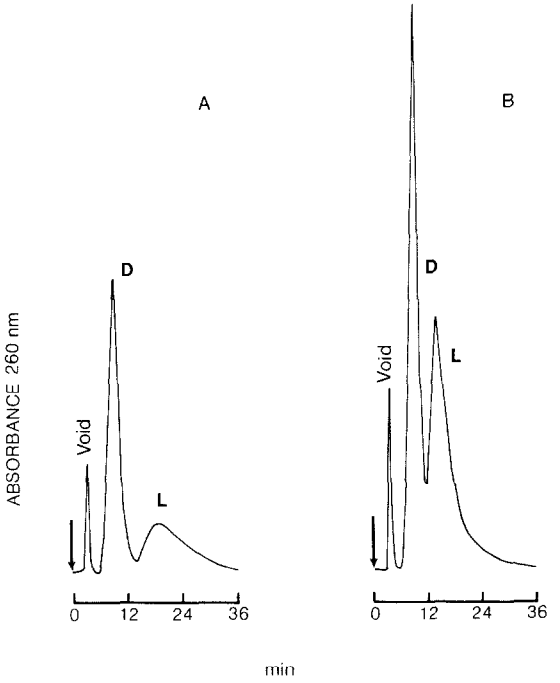

Fig. 1. Resolution of the enantiomers of the print molecule. Particles $< 25\ \mu m$ were packed into a 200 mm × 5 mm stainless-steel column. Analyses were performed at room temperature under isocratic conditions using acetonitrile–acetic acid (9:1, v/v) as the eluent at a flow-rate of 1 ml/min. Detection was at 260 nm. Samples consisted of a mixture of either 5 (A) or 100 μg (B) of each of the enantiomers of phenylalanine anilide. Boc-L-Phenylalanine anilide was included in each sample as a non-interacting, non-excluded void marker. The arrows indicate the point of sample injection. Peaks corresponding to the void and the D- and L-enantiomers of phenylalanine anilide are clearly indicated.

tiation with AIBN at 0°C and chloroform as the polymerization solvent. As is seen, baseline separations were obtained with low sample loads (5 μg of each enantiomer; A). The polymer also exhibited a rather high capacity in that acceptable separations were obtained with sample loads of 100 μg of each enantiomer (B). It should be noted that the results presented in Fig. 1 were obtained under isocratic conditions at room temperature. This contrasts with previous reports where separations needed to be performed at column temperatures of up to 90°C[10].

As has been described for other HPLC matrices, the use of small particles and long, thin columns, increased the separations obtained on molecularly imprinted polymers (data not shown). Routinely therefore, particles of < 25 μm and columns of 200 mm × 5 mm were used in subsequent experiments.

A previous report suggested that the functional monomer, methacrylic acid, interacts with the print molecule, phenylalanine anilide, in a 2:1 complex involving an ionic bond to the free primary amine and an hydrogen bond to the amide[10]. We were interested in defining these interactions from the point of view of polymer preparation and specificity. Therefore, a series of polymers, listed in Table II, were prepared and analysed in HPLC for their ability to separate the enantiomers of phenylalanine anilide. Polymer 1, prepared in the absence of print molecule, and polymer 2, prepared using a non-interacting monomer (methyl methacrylate), did not retain phenylalanine anilide. This result shows that recognition of molecules by molecularly imprinted polymers depends on both the print molecule and the functional monomer and is therefore a "specific" event. Polymer 3 was also unable to recognize phenylalanine anilide. This indicates that a one-point interaction between the print molecule and the polymer is insufficient for enantiomeric resolution, at least in this system and under the conditions used for analysis. The results of analyses of polymers 4–6 indicated that the "optimum" ratio of functional monomer/print molecule was 4:1 (not shown).

Enantiomeric resolution of other amino acid amides

We considered that a polymer prepared using phenylalanine anilide as the print molecule may also be able to separate the enantiomers of other amide derivatives of amino acids, since the positioning of functional groups within the polymer was de-

TABLE II

POLYMER PREPARATIONS

Polymer[a] *(monomer:print)*	*mmol component added*[b]			
	PA	*MAA*	*MM*	*EDMA*
1 (0)	0	7.5	0	36.62
2 (0)	1.956	0	7.86	39.3
3 (1:2)	1.956	0.975	6.885	39.3
4 (2:1)	1.956	3.93	3.93	39.3
5 (4:1)	1.956	7.86	0	39.3
6 (8:1)	1.31	10.48	0	50.26

[a] (monomer:print) refers to the molar ratio of functional monomer (MAA) to print molecule (PA).

[b] PA = L-Phenylalanine anilide; MAA = methacrylic acid; MM = methyl methacrylate; EDMA = ethylene glycol dimethacrylate.

TABLE III

ENANTIOMERIC RESOLUTION OF AMIDE DERIVATIVES OF AMINO ACIDS ON POLYMER 5 (TABLE II)

A mixture of 25 μg of each of the enantiomers of the amide derivative of the amino acid was injected into the column in a total volume of 20 μl and eluted with acetonitrile–acetic acid (9:1, v/v) at room temperature. Detection was at 260 nm. The (+) indicates that enantiomeric resolution was obtained. NT indicates that the compound was not tested, either because both enantiomers were not commercially available or UV detection was not possible, *e.g.*, for the amides of leucine and alanine.

Amino acid	*Derivative*[a]			
	A	*p-NA*	*β-NA*	*Amide*
Phenylalanine	(+)	(+)	NT	(+)
Tyrosine	(+)	NT	NT	NT
Tryptophan	(+)	NT	NT	(+)
Leucine	NT	(+)	(+)	NT
Alanine	NT	(+)	(+)	NT

[a] A = Anilide; *p*-NA = *p*-nitroanilide; β-NA = β-naphthylamide.

fined. A number of derivatives of amino acids, including amides, esters and dipeptides, were thus analysed on polymer 5 (Table II). In all cases investigated, enantiomeric resolution was obtained for the amide derivatives of amino acids (see Table III) and representative elution profiles are shown in Fig. 2.

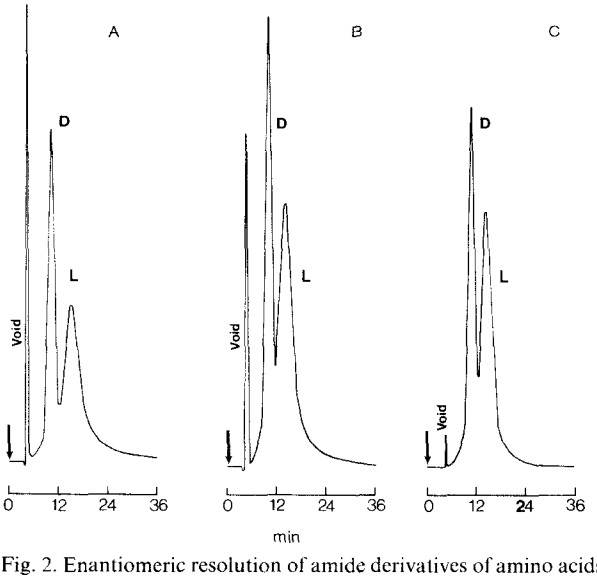

Fig. 2. Enantiomeric resolution of amide derivatives of amino acids on polymer 5 (Table II). Particles < 25 μm, prepared from polymer 5 (Table II), were packed into a 200 mm × 5 mm column. Analyses were performed at room temperature with acetonitrile–acetic acid (9:1, v/v) as the eluent at 1 ml/min. Detection was at 260 nm. In all experiments, a mixture of 25 μg of each of the enantiomers of the compound was analysed. Boc-L-Phenylalanine anilide was included as a void marker. The analyses shown are: (A) D,L-tryptophan anilide; (B) D,L-leucine-β-naphthylamide; (C) D,L-alanine-*p*-nitroanilide.

From these data it is clear that a single polymer is able to recognize and separate enantiomers of molecules having structures similar to that of the original print molecule. The most important factor for enantiomeric resolution in this system would appear to be the presence of both an amine and an amide, in the correct spatial orientation, on the molecule being analysed. This is clearly demonstrated by the fact that removal of the amine, as in Boc-L-phenylalanine anilide, results in elution of this compound with the solvent front (see Figs. 1 and 2). In addition, removal of the amide, as in D,L-phenylalanine benzyl ester, results in retention but no enantiomeric resolution (not shown).

Preliminary experiments also indicate that the enantiomers of phenylalanine-containing dipeptides such as D,L-phenylalanylglycine ethyl ester are resolved on this polymer, whereas glycyl-D,L-phenylalanine is retained but no enantiomeric resolution is obtained. Therefore, polymers prepared by the technique of molecular imprinting may find use in the identification and purification of peptides.

Proposed mechanism of enantiomeric resolution

Both a free primary amine and an amide, in the correct spatial orientation, are required to achieve enantiomeric resolution. Initial recognition probably occurs via "ion pairing" between the free primary amine of the molecule and a free carboxylic acid group of the polymer. This relatively strong interaction places the molecule in a position to hydrogen bond through the amide function to another carboxylic acid function of the polymer. Since polymers prepared in the absence of either print molecule or functional monomer (polymers 1 and 2, Table II) do not subsequently recognize the print molecule, it can be concluded that enantiomeric discrimination by polymers, such as polymer 5, is a specific event and dependent on the interaction(s) of print molecules with functional monomers in solution prior to polymerization. These interactions result in a polymer which has carboxylic acid functions exactly positioned to interact with the print molecule in a stereospecific manner. In addition, the mechanism of recognition cannot be of a simple ion-exchange type, since this would also be expected to occur in a polymer with randomly distributed carboxylic acid functions, as in polymer 1 (Table II). Therefore, it can be assumed that the stereospecific interaction of molecules with the polymer occurs within specific sites of the polymer which are a direct result of the interactions of molecules in solution prior to polymerization.

DISCUSSION

The technique of molecular imprinting is gaining acceptance as a means of producing specialty separation media, particularly for enantiomeric resolution of chiral molecules[1]. In the present paper, we have presented some of our recent work on the preparation of molecular imprints for the enantiomeric resolution of amino acid derivatives. The method involves the formation of non-covalent interactions between the print molecule and functional monomers, in solution, prior to the initiation of polymerization. These interactions are subsequently responsible for the recognition of molecules by the polymer.

The method of polymerization presented herein shows distinct advantages over those previously presented from the point of view of simplicity and speed of prep-

aration of polymers. From an operational standpoint, polymers prepared by this technique exhibit excellent enantiomeric resolution under standard HPLC conditions. HPLC analyses were performed at room temperature and at flow-rates of 1 ml/min, resulting in pressures of 60—70 bar for a 200 mm × 5 mm column, packed with particles < 25 μm. Previous descriptions of the use of molecular imprints in the HPLC mode required column temperatures of up to 90°C, and flow-rates of only 0.3 ml/min were used to achieve separations comparable to those presented here[1,4,10]. In addition, the demonstration that efficient molecular imprints can be prepared simply at temperatures down to 0°C adds a degree of flexibility to the method of preparation of polymers and should be particularly valuable for the imprinting of temperature-sensitive molecules. However, it is important to note that, even in the system studied here, better printing was obtained with polymerization at 0°C than at 60°C. Therefore, the preparation of molecular imprints at low temperature should be considered as a general method. It should also be stressed that the initiator used in the present system, AIBN, is the same initiator used by previous workers for the preparation of molecular imprints at temperatures ranging from 60 to 120°C[1,10].

The interactions between the print molecule and functional monomer prior to polymerization are, of course, central to the successful preparation of molecular imprints. Two factors affecting these interactions are the temperature (discussed above) and solvent of polymerization. Similar to work of other investigators[1] we have shown that it is possible to "tailor" the strength of the interactions by manipulation of the solvent of polymerization[11]. This may prove useful in certain situations. Of course, the same effect(s) of solvent should apply to the elution of compounds from molecularly imprinted polymers. However, little work has been done in this area. It is possible that a thorough investigation of elution conditions may further improve the separations, particularly the peak shape. It should be noted that extremely irregular particles of relatively poorly defined size have been used for the analysis of molecular imprints. The preparation of molecular imprints in a beaded form, with small particle size, as is common for other HPLC packings, may also prove advantageous.

The ability of a single polymer to separate the enantiomers of molecules other than the print molecule, as presented here, is an important concept. This may allow the design and preparation of molecular imprints by using a "soluble" derivative of a molecule which is itself insoluble in the polymerization mixture. An example of this is D,L-phenylalanine amide, which is insoluble in the polymerization mixture but may be applied and separated on a polymer prepared using L-phenylalanine anilide as the "soluble" print molecule. We are following this approach for the imprinting of molecules other than amino acid derivatives. Another potential application of molecular imprinting is in preparative-scale chiral separations of biological or pharmaceutical preparations. This would be of particular value in situations where other chiral separation methods do not achieve the required enantiomeric enrichment.

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