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Journal of Chromatography A, 775 (1997) 51–63

JOURNAL OF
CHROMATOGRAPHY A

Molecularly imprinted ligand-exchange adsorbents for the chiral separation of underivatized amino acids

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Received 20 January 1997; revised 10 March 1997; accepted 10 March 1997

Abstract

Ligand-exchange adsorbents that are enantioselective for underivatized α-amino acids have been synthesized by molecular imprinting using only achiral monomers. Bulk polymers were prepared by allowing the functional monomer, Cu(II)–N-(4-vinylbenzyl)iminodiacetic acid, to form complexes with the template amino acid in solution, followed by crosslinking with ethylene glycol dimethacrylate. To make supports suitable for chromatography, the imprinted polymer was grafted to derivatized silica particles. Racemic mixtures of various underivatized α-amino acids are resolved on the imprinted adsorbents. Adsorbents prepared from amino acids with larger, aromatic side chains exhibit the highest selectivities ($\alpha=1.65$ for the enantioresolution of D,L-phenylalanine). Cross-selectivity for similar amino acids also depends on side chain size: materials templated with L- or D-phenylalanine exhibit good enantioselectivity when challenged with racemic tyrosine ($\alpha \sim 1.4$) and much reduced enantioselectivities towards D,L-tryptophan or aliphatic amino acids. Materials imprinted with alanine show no selectivity. The ability of a material imprinted with an amino acid enantiomer to resolve an analogous chiral amine is also demonstrated. The mechanisms underlying the observed enantioselectivity are discussed in light of the three-point interaction model for conventional chiral ligand-exchange separations. © 1997 Elsevier Science B.V.

Keywords: Molecular imprinting; Enantiomer separation; Ligand-exchange chromatography; Chiral stationary phases, LC; Amino acids; Metal chelates

1. Introduction

Ligand-exchange chromatography (LEC) exploits the rapid and reversible formation of metal ion complexes to separate compounds which can donate electrons and coordinate to the immobilized metal ions [1]. Solvent components occupying coordination sites on the metal centers are displaced by ligands from the sample. Retention of a given species is directly related to the stability of the mixed ligand complex it forms with the metal ion complex immobilized on a chromatographic support. Ligand exchange with soluble metal complexes that partition between the mobile and solid phases also affects retention.

LEC has been successfully applied to the chiral separation of a large number of amines [1], carbohydrates [2] and various β-blocker and adrenergic drugs that contain the amino alcohol functionality [3]. One particularly well-studied application of LEC is the chiral separation of amino acids [1,4,5]. Chiral stationary phases (CSPs) for LEC of amino acids are typically synthesized by covalent attachment of a chiral metal-chelating ligand such as L-proline to a

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PII S0021-9673(97)00280-X

chromatographic support. A polymerizable derivative of L-proline can be used, for example, as a comonomer in a crosslinking polymerization [4], or it can be grafted onto the surface of a silica support [5]. During chromatography the mixed ligand complexes that form at the adsorbent surface have two chiral ligands coordinated to the metal ion, the L-proline from the CSP itself and an enantiomer from the sample. The complex is therefore a diastereomeric adduct, the two forms of which are expected to have different stabilities and are therefore resolved in the chromatographic separation. Formation of a diastereomeric adduct, however, is not sufficient to result in a separation. The interaction between the solid support and the mixed ligand complex is important in determining the resolution, and the retention order of enantiomers on such materials is not always predictable. For example, polystyrene–divinylbenzene derivatized with L-proline [1] shows a different order of elution as compared to a polyacrylamide-based material [4] for chiral separation of D,L-phenylalanine. The choice of adsorbent is a matter of trial and error, as is the optimization of the separation protocol.

Our laboratory is developing a different, and conceivably generalizable, approach to preparing chirally selective ligand-exchange supports using the technique of molecular imprinting. In molecular imprinting, the target molecule serves as a template to assemble complementary recognition sites in a crosslinked polymer. The resulting materials selectively bind the templates with which they were prepared [6–8]. To prepare the adsorbent, the functional monomer (or mixture or monomers) is generally allowed to complex with the template in solution. Formation of the monomer–template assembly is followed by polymerization carried out in the presence of (excess) crosslinking comonomer. The polymer is expected to contain binding sites selective for the template molecule over other similar, competing substrates. Molecular imprinting is attractive for preparing selective adsorbents for chiral and other separations for which conventional adsorbents might be unsuitable [6–8].

Preorganization of the monomers and template may be achieved using various interactions, including hydrogen bonding [6], covalent bonding [7], and metal ion coordination [9]. Molecular imprinting with hydrogen-bonding (methacrylic acid) monomers has been used by several groups to prepare chiral adsorbents capable of separating amino acid derivatives [10–13]. Derivatives such as L-phenylalanine anilide or *tert.*-butyloxycarbonyl-L-phenylalanine were used as the templates, and the resulting adsorbents could separate only the derivatized amino acids. Derivatization helps to eliminate repulsive electrostatic interactions between methacrylic acid and the underivatized amino acid; the bulky side group also assists chiral recognition. The obvious disadvantages are that derivatization is cumbersome and additional steps are required to retrieve the free amino acid after separation. Also, reliance on hydrogen bonding for retention imposes a further limitation in that amino acid enantioresolution on such materials is difficult or impossible in aqueous media. Finally, it is clear that weak interactions in the preorganization step lead to random incorporation of the functional monomer and loss of selectivity.

The use of metal ion complexes in molecular imprinting to prepare selective ligand-exchange adsorbents is largely unexplored [8,15,16]. Metal coordination–chelation interactions offer important advantages over other interactions for molecular imprinting. Metal coordination–chelation interactions are strong relative to hydrogen bonding, especially in water, and therefore can significantly reduce the random incorporation of functional monomer. Binding strength and kinetics of binding can be tuned through the choice of metal ion, which can be substituted easily once the adsorbent has been prepared [16,17]. In this study, we have combined molecular imprinting with the ability of amino acids to chelate metal ions to create LEC supports suitable for chiral separations of underivatized amino acids. We have investigated several aliphatic and aromatic amino acids in order to evaluate the role of the side group in imparting enantioselectivity. By working with an achiral monomer based on iminodiacetate, we show that the enantioselectivity of the adsorbent arises from the chirality of recognition sites created during polymerization.

2. Experimental

2.1. Materials

All materials were of reagent grade and obtained

from Aldrich, except for α-methylphenethylamine sulfate, which was purchased from Chem Service (Westchester, PA, USA).

2.2. Instrumentation

Elemental analyses were carried out at Galbraith Labs. (Knoxville, TN, USA). Electronic absorption spectroscopic measurements were carried out with a Milton Roy 3000 spectrophotometer. Isothermal titration calorimetry was carried out on a Microcal calorimeter and the data analyzed using software supplied by Microcal (Northamptom, MA, USA). Optical activity measurements were carried out using a Perkin–Elmer 300 polarimeter equipped with a sodium lamp. Chromatography experiments were carried out in a 50 mm×4.6 mm I.D. column using a Hitachi HPLC system with a L-6200 pump and UV detection at 270 nm using a Kratos Spectroflow 783 detector.

2.3. Preparation of monomer–metal–amino acid complex

Preparation of the complex involved two steps: isolation of Cu(II)–[N-(4-vinylbenzyl)imino]diacetate [Cu(VBIDA)] followed by the addition of amino acid. Here we describe the preparation of the ternary complex with phenylalanine. Mixed-ligand complexes incorporating the amino acids tyrosine, leucine, isoleucine, valine and alanine were obtained using similar procedures.

VBIDA was synthesized as reported previously [9]. However, the Cu(II) complex of VBIDA was prepared using a modification of the previously reported procedure. VBIDA (5.00 g) was dissolved in 150 ml distilled water. The pH was adjusted to 9.5 using 6 *M* NaOH. $CuSO_4 \cdot 5H_2O$ (5.00 g) dissolved in 150 ml distilled water, was added dropwise to the VBIDA solution, adjusting the pH with 1 *M* NaOH to maintain its value at 9.5. This high dilution was necessary to keep all species in solution as the $CuSO_4$ solution was titrated into the VBIDA solution. The high pH helped to keep the IDA ligand deprotonated for chelation to the Cu(II) ion as it was titrated in. In order to avoid irreversible precipitation of Cu(II) as a hydroxide species, the $CuSO_4$ solution was added dropwise. The chelation of Cu(II) by VBIDA could be seen by the shift in the color of the solution to a dark blue, and the binding reaction was also followed by UV–Vis spectroscopy (λ=700 nm).

The resulting dark blue solution was vacuum filtered and diluted to 500 ml with distilled water. The solution was frozen at −70°C and lyophilized. The lyophilized powder was dissolved in 100 ml of 100% methanol and stirred for 1 h. The methanol solution was vacuum filtered and the solvent removed by rotary evaporation. The solid obtained was then redissolved in 50 ml 100% methanol and stirred for 1 h. The solution was filtered and the solvent removed by rotary evaporation of the filtrate, after which the desired complex was obtained as a dark blue solid: yield=62%. At high pH, the Cu(II) complex of VBIDA was isolated as the Na salt of Cu(VBIDA)(OH)(H_2O). Anal.: Calcd. for $C_{13}H_{16}O_6NCuNa$: C, 42.34; N, 3.8; H, 4.37; Cu, 17.23. Found: C, 42.55; N, 3.77; H, 4.42; Cu, 17.20.

Formation of the Cu(VBIDA)(phe) complex was achieved by titrating the amino acid solution into a solution of the Cu(VBIDA) complex. A 2-g amount of the purified Cu(VBIDA) was dissolved in distilled water to make a 20 m*M* solution. A 1.4-g amount [2 molar equivalents, as suggested by the isothermal titration calorimetry (ITC) experiments] of phenylalanine was dissolved in 30 ml of distilled water and added dropwise to the Cu(VBIDA) solution, maintaining the pH between 9 and 9.5 using 1 *M* NaOH. The solution was stirred for 1 h and subsequently cooled to −70°C and lyophilized (yield=84%). The ternary metal complex incorporating the amino acid was also isolated as the sodium salt as the reaction was done at high pH. Anal.: Calcd. for $C_{22}H_{23}O_6NCuNa$: C, 58.61; H, 5.14; N, 6.21; Cu, 14.09. Found: C, 58.59; H, 5.09; N, 6.19; Cu, 14.03. Complexes using racemic phe, D-phenylalanine and L-phenylalanine were prepared by the above method, to obtain Cu(VBIDA)(*rac*-phe), Cu(VBIDA)(D-phe) and Cu(VBIDA)(L-phe), respectively. Mixed ligand complexes incorporating the amino acids alanine, valine, leucine, isoleucine and tyrosine were prepared using identical procedures.

2.4. Isothermal titration calorimetry

The preorganization of the Cu(VBIDA) monomer with phenylalanine was followed by ITC. In this experiment, a 1 m*M* solution of Cu(VBIDA) was prepared at a pH of 9.5, and 1 ml of this solution was

loaded into the calorimetry sample cell, which was equilibrated at 25°C. A 250-μl volume of a 20 m*M* solution of the phenylalanine solution at the same pH was titrated into the sample cell, the volume of each addition being 10 μl. The reference cell was loaded with distilled water that had been brought to a pH of 9.5 by addition of NaOH. The binding of phenylalanine to the Cu(VBIDA) to form the mixed ligand complex is exothermic, and the power required to maintain the sample cell at the same temperature as the reference cell was measured. The data were fit to a one-site model using software supplied by Microcal to calculate the binding constant of phenylalanine to Cu(VBIDA).

2.5. Polymerization and workup

In a typical polymerization reaction, 0.5 g of the MMA complex was dissolved in 5 ml of water, and the solution is stirred for 6 h under nitrogen atmosphere. An excess of ethylene glycol dimethacrylate (~95 mol%) and 4,4′-azobis(4-cyanovaleric acid) (1%, w/w, with respect to total monomers), dissolved in 15 ml of methanol, were added to this solution. The polymerization mixture was cooled to liquid nitrogen temperature, evacuated, thawed, and then purged with nitrogen. This procedure was repeated thrice to remove oxygen, and polymerization was carried out at 40°C for 48 h under nitrogen. The solid polymer thus obtained was cooled, ground, and extracted thoroughly with methanol to remove unreacted monomers and crosslinkers. The resulting blue polymers were then dried to constant weight at 50°C under vacuum and sieved to an appropriate particle size. Particles between 10 μm and 50 μm in size were used for the equilibrium rebinding experiments.

2.6. Derivatization of silica particles

In order to prepare imprinted materials suitable for chromatographic separations, the polymers were also synthesized as surface coats on silica particles, following procedures used previously in this laboratory [16]. Silica (LiChrospher 1000, 10 μm particles, 1000 Å pores, supplied by E. Merck, Darmstadt, Germany) was washed with distilled water and boiled in 5% HNO_3. The particles were filtered on a fine-fritted filter, washed extensively with distilled water, and dried at 150°C for 24 h. A 1-μm vacuum was applied to the oven-dried silica in a three-neck flask for 30 min, after which the vessel was sealed. Toluene, dried over sodium and distilled, was added to the silica under vacuum. 3-(Trimethoxysilyl)propyl methacrylate (50%, w/w, silica) and a trace of triethylamine were added to complete the reaction mixture. This mixture was refluxed for 15 h under nitrogen. The silica was isolated by filtration over a fine-fritted filter and freed of any residue by successive washing with toluene, acetone, and diethyl ether. The derivatized silica was then dried to constant mass under vacuum for 24 h.

2.7. Polymer coating process

A typical procedure for preparing templated polymers using Cu(VBIDA)(D-phe) is outlined below. Identical procedures were used for synthesis of materials with L-amino acid and *rac*-amino acid as templates. Propylmethacrylate-derivatized silica (4 g) was placed in a 50-ml three-necked round-bottomed flask and a 1-μm vacuum applied. Methanol–water (80:20, v/v) was added under vacuum to cover the surface (about 10 ml). Ethylene glycol dimethacrylate (EGDMA) (0.76 g) and Cu(VBIDA)(D-phe) (0.07 g) were then added to the silica particles under vacuum. After mixing for 1 h, this mixture was sonicated for 20 min to allow penetration of solvent into the pore spaces of the silica particles. The initiator, 4,4′-azobis(4-cyanovaleric acid) (10 mg) was dissolved in 5 μl of methanol and added under vacuum, after which the vessel was sealed. The reaction mixture was then placed in a constant temperature shaker bath at 40°C for 48 h with gentle agitation. The coated silica was then washed thoroughly with methanol to extract out the unreacted monomers, following which it was dried to constant mass at 50°C under vacuum.

2.8. Template and Cu(II) removal and reloading

Removal of both the amino acid template and Cu(II) was achieved by equilibrating the imprinted polymers with 1 *M* ethylenediaminetetraacetic acid

(EDTA) at pH 8 for 48 h. The amount of Cu(II) removed was determined by UV–Vis spectroscopy.

The polymers were subsequently washed three times with 50-ml portions of distilled water in order to remove residual EDTA and template molecules. Then enough water was added to immerse the polymer particles and a few drops of 1 *M* NaOH were added until the pH stabilized to reach a final value of 8. The mixture was then centrifuged and the polymer was filtered off. To reload the polymers with Cu(II), 20 ml of a 0.5 *M* solution of $CuSO_4$ was added. After equilibration overnight, the polymer was washed thrice with 50-ml portions of distilled water (until solution was no longer blue), saving the supernatants. The amount of Cu(II) in the supernatant was determined as $[Cu(EDTA)]^{2-}$ by UV–Vis spectroscopy and the reloading capacity calculated. Reloading was nearly quantitative with at least 98% of Cu(II) reloaded, based on the amount of Cu(II) removed earlier from the material. Similar procedures were followed for the workup of the polymer coated silica materials.

2.9. Polarimetric analysis – equilibrium rebinding

In a typical equilibrium rebinding experiment, 50 mg of amino acid, e.g. D,L-phe, were dissolved in 10 ml of distilled water. The amino acid solution was equilibrated to a pH of 9 with a few drops of 1 *M* NaOH and added to 1 g of the imprinted polymer to equilibrate for 24 h, following which the supernatant was decanted out after centrifugation. The silica was then washed thoroughly with 25 ml of distilled water, followed by another centrifugation and the supernatant was decanted. The total phenylalanine concentration in the supernatant solution was determined using UV–Vis spectroscopy. The combined supernatant was then freeze-dried to a smaller volume of 10 ml for polarimetry analysis. Optical rotation was observed at 25°C using a sodium lamp at 589 nm. The observed optical purity of the supernatant, combined with a mass balance, was used to calculate equilibrium rebinding selectivity.

2.10. Chromatography

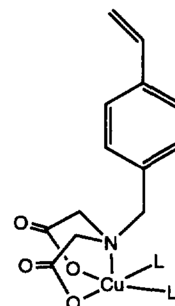
The Cu-free polymer-coated silica was packed into a chromatography column (50 mm×4.6 mm I.D.) following procedures developed previously [16]. Cu(II) was reloaded onto the column by washing with 20 m*M* $CuSO_4$ solution at a flow-rate of 1 ml min^{-1}, followed by washing with water until a steady baseline was reached. A 100-μl volume of a 1 m*M* solution of racemic amino acid was used as the sample loading in a typical experiment. Chromatographic separations were achieved at 50°C, using isocratic elution with 1.5 m*M* glycine for the elution of amino acid enantiomers. The elution of α-methylphenethylamine and α-methylhydroxycinnamic acid was carried out isocratically with 1.5 m*M* acetate at pH 8, 50°C.

3. Results and discussion

3.1. Preparation of monomer–metal–amino acid complexes

Monomer-template preorganization involves formation of a mixed ligand complex of metal-complexing monomer and the pure amino acid enantiomer that will serve as the template. The metal-complexing monomer chosen for this study, Cu(II)–[N-(4-vinylbenzyl)imino]diacetic acid [Cu(VBIDA)], is achiral and cannot itself distinguish between L- and D-amino acids (Fig. 1).

Phenylalanine, tyrosine, alanine, valine, leucine, and isoleucine were used as templates. The association constants for these amino acids to Cu(IDA) in aqueous solution lie between 10^3 to 10^4 M^{-1} [18].



Cu(II)[N-(4-vinylbenzyl)]iminodiacetate

Fig. 1. The achiral functional monomer, Cu(II)–[N-(4-vinylbenzyl)]iminodiacetate [Cu(VBIDA)].

Formation of the ternary monomer–metal–template complex of phenylalanine to Cu(VBIDA) was monitored at pH 9.5 by ITC. The ITC experiments were carried out in the same concentration range used for subsequent polymerization reactions (~1 m*M* for Cu(VBIDA)). The ITC data indicate that the binding between Cu(VBIDA) and L-phenylalanine reaches saturation only when more than two equivalents of amino acid are added (Fig. 2) and the binding constant is 2.7 (±0.3)·10^4 M^{-1}. The ITC data also confirm that metal is not stripped out into metal–amino acid complexes at these concentrations. The binding of the amino acid is not strong enough to compete with the IDA monomer for metal ions (binding constant for Cu(II) to IDA is ~10^{11} M^{-1}) [19]. The titration behavior of D-phenylalanine is identical to that of the L-enantiomer, as expected for achiral Cu(VBIDA) (data not shown). In order to minimize the random incorporation of functional monomer into the polymer matrix in preparing the imprinted materials, a greater than two-fold excess of template amino acid was used during polymerization. Under these conditions of excess template, some of the template remains unbound, but essentially all the Cu(VBIDA) monomer is in a 1:1 complex with amino acid.

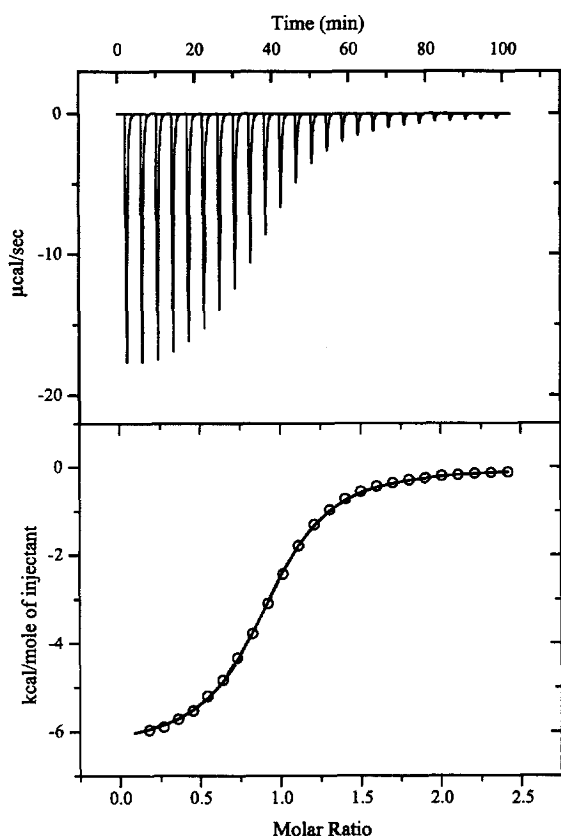


Fig. 2. ITC data for binding of phenylalanine to Cu(VBIDA). The ITC cell was loaded with a 1 m*M* Cu(VBIDA) solution at 25°C (pH 9.5). A 250-µl volume of a 20 m*M* solution of the amino acid solution at the same pH was titrated into the ITC cell (addition volume=10 µl). The one-site binding model used to fit the data gave a binding constant of 2.7 (±0.3)×10^4 M^{-1} for the binding of L-phenylalanine to Cu(VBIDA). Identical results were obtained for the binding of D-phenylalanine to Cu(VBIDA).

3.2. Polymerization and workup

The strategy for preparing molecularly-imprinted ligand-exchange materials is outlined in Fig. 3. The metal-complexing monomer [Cu(VBIDA)] and the amino acid template (e.g. D-phenylalanine) are pre-organized in solution to form the monomer–metal–amino acid complex, as described above. Cross-linking polymerization with ethylene glycol dimethacrylate (EGDMA) with thermal initiation using 4,4′-azobis(4-cyanovaleric acid) in methanol–water (80:20, v/v) creates a macroporous polymer matrix. A 5:95 molar ratio of functional monomer to crosslinker was used. In order to make materials suitable for column chromatography, the polymers imprinted using phenylalanine enantiomers were also synthes-

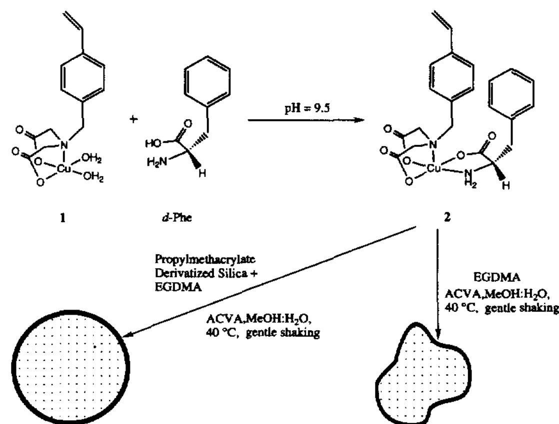


Fig. 3. Molecular imprinting of a phenylalanine enantiomer to form (a) a polymer coating on the surface of silica particles or (b) a bulk polymer.

ized as surface coatings on spherical silica particles (LiChrospher, 10-μm size, 1000 Å pores) derivatized with 3-(trimethoxysilyl)propyl methacrylate, using procedures described previously [16].

For each amino acid studied, the following bulk polymers were prepared: polymers imprinted against each enantiomer and a control polymer synthesized using the racemic amino acid as the template, which should show no enantioselectivity. A further control polymer was also synthesized with no template. The bulk polymers were washed thoroughly with water, ground and filtered. The washes were combined, the solvent was removed by lyophilization, and the residual solids were carefully weighed. After drying in a vacuum oven at 60°C, the polymers were weighed again and then treated with ethylenediaminetetraacetic acid (EDTA) to remove the metal ions and template. The amount of Cu(II) removed was estimated by UV–Vis spectroscopy. The metal removal step was followed by thorough washing, and the materials were dried in a vacuum oven and weighed again. These gravimetric measurements and the UV–Vis spectroscopic estimation of Cu allowed us to calculate the amounts of template removed. For all the amino acids studied, the template removal was never less than 97% of the total amino acid used in the polymerization scheme Thus, after a typical polymer workup procedure, the amount of reversibly bound metal (and template) was estimated to be 25 $\mu mol\ g^{-1}$ polymer (dry mass), whereas the amount of residual, irreversibly bound template was always less than 0.8 $\mu mol\ g^{-1}$. The dried polymers were sieved, and a size fraction between 10 μm and 50 μm was used for equilibrium rebinding experiments, both in the metal-free form and after reloading with copper ions. Reloading of Cu(II) was achieved by equilibration with a 100 m*M* solution of $CuSO_4$.

3.3. Equilibrium rebinding

Competitive equilibrium rebinding studies were carried out to determine the enantioselectivities of binding for different amino acids at pH 9. In a typical rebinding experiment, polymer reloaded with Cu(II) was equilibrated with an aqueous solution containing a two-fold excess of the racemic amino acid (5 m*M*, pH 9) for 24 h, after which the supernatant was recovered by filtration. Polarimetry was used to determine the optical activity of the supernatant solution, and an enantiomeric excess was calculated by combining this measurement with UV-absorption measurement of the total amino acid concentration. The Cu-free materials showed negligible rebinding to all amino acids, indicating that there is negligible non-specific binding under these conditions.

Table 1 summarizes the results of these experiments. For each amino acid studied, the control materials prepared using racemic template and using no template showed no preference for either enantiomer. In contrast, the materials imprinted with a single enantiomer are selective for that enantiomer. The highest enantioselectivities were seen for the polymers imprinted with tyrosine and phenylalanine ($\alpha \sim 1.45$). Materials imprinted with L- or D-alanine showed little enantioselectivity for any of the amino acids studied, including alanine. Polymers imprinted using D- and L-valine showed low enantioselectivities for valine ($\alpha \sim 1.08$) and no selectivity for enantiomers of any of the other amino acids.

Table 1 also shows that the materials templated with L- or D-phenylalanine exhibit good enantioselectivity ($\alpha \sim 1.4$) when challenged with racemic tyrosine and much reduced enantioselectivities when equilibrated with D,L-tryptophan ($\alpha \sim 1.1$). Little or no selectivity was seen for alanine, valine, leucine or isoleucine. Similarly, materials imprinted with L- or D-tyrosine can distinguish the enantiomers of phenylalanine and tryptophan, but not the aliphatic amino acids. Materials imprinted with a leucine or an isoleucine enantiomer exhibit cross-enantioselectivity for the other amino acid and reduced ability to resolve phenylalanine. No selectivity was seen for alanine or valine.

A consistent trend to higher enantioselectivity with increasing size of the side group is evident, indicating that the size of the amino acid side chain is an important factor in determining enantioselectivity in the molecularly-imprinted ligand-exchange polymers. The imprinted polymers also show interesting cross-selectivity patterns. Tyrosine with its phenolic side chain differs only slightly from phenylalanine in molecular size, and polymers imprinted with these templates show comparable enantioselectivities in rebinding the other amino acid. Similarly, leucine and isoleucine are quite similar in size, and the

Table 1
Enantioselectivities of molecularly imprinted polymers in equilibrium rebinding experiments

Polymers	Enantioselectivity for amino acids[a]						
	D/L-Alanine	D/L-Valine	D/L-Leucine	D/L-Isoleucine	D/L-Phenylalanine	D/L-Tyrosine	D/L-Tryptophan[b]
L-Amino acid template	$\alpha_{L/D}$						
P–L-A	1.02 (±0.01)	1.01 (±0.01)	1.01 (±0.01)	0.98 (±0.03)	0.99 (±0.03)	1.00 (±0.02)	–
P–L-V	1.00 (±0.01)	*1.08* (±0.01)	1.01 (±0.02)	1.00 (±0.01)	1.01 (±0.01)	1.02 (±0.01)	-
P–L-L	0.99 (±0.01)	1.03 (±0.01)	*1.19* (±0.01)	*1.14* (±0.01)	1.07 (±0.01)	1.06 (±0.03)	–
P–L-I	0.99 (±0.03)	1.02 (±0.02)	*1.18* (±0.01)	*1.23* (±0.01)	1.08 (±0.03)	1.07 (±0.02)	–
P–L-F	0.98 (±0.01)	1.01 (±0.01)	1.04 (±0.02)	1.07 (±0.02)	*1.45* (±0.01)	*1.38* (±0.01)	*1.12* (±0.01)
P–L-Y	1.01 (±0.03)	1.00 (±0.01)	1.05 (±0.02)	1.08 (±0.02)	*1.36* (±0.01)	*1.42* (±0.01)	*1.14* (±0.01)
D-Amino acid template	$\alpha_{D/L}$						
P–D-A	1.01 (±0.01)	0.99 (±0.01)	0.99 (±0.01)	1.00 (±0.03)	0.98 (±0.02)	1.00 (±0.02)	–
P–D-V	1.01 (±0.01)	*1.09* (±0.01)	1.00 (±0.02)	0.99 (±0.01)	1.00 (±0.02)	1.02 (±0.01)	–
P–D-L	1.00 (±0.01)	1.01 (±0.01)	*1.15* (±0.01)	*1.12* (±0.01)	1.08 (±0.01)	1.07 (±0.01)	–
P–D-I	1.01 (±0.03)	1.03 (±0.02)	*1.16* (±0.01)	*1.24* (±0.04)	1.08 (±0.03)	1.06 (±0.02)	–
P–D-F	0.99 (±0.01)	1.00 (±0.01)	1.05 (±0.02)	1.07 (±0.03)	*1.47* (±0.01)	*1.39* (±0.01)	*1.13* (±0.01)
P–D-Y	1.00 (±0.02)	0.99 (±0.01)	1.04 (±0.01)	1.08 (±0.01)	*1.36* (±0.01)	*1.43* (±0.02)	*1.12* (±0.01)
Controls	$\alpha_{L/D}$						
P–D,L-A	1.01 (±0.01)	1.00 (±0.03)	1.00 (±0.01)	1.00 (±0.03)	0.99 (±0.03)	1.00 (±0.02)	–
P–D,L-V	0.99 (±0.03)	1.01 (±0.01)	1.00 (±0.01)	1.01 (±0.01)	1.09 (±0.01)	1.01 (±0.01)	–
P–D,L-L	1.00 (±0.01)	0.99 (±0.02)	1.01 (±0.01)	0.98 (±0.02)	1.01 (±0.01)	1.00 (±0.01)	–
P–D,L-I	1.01 (±0.01)	1.00 (±0.02)	1.00 (±0.01)	0.99 (±0.01)	0.98 (±0.02)	0.99 (±0.02)	–
P–D,L-F	0.98 (±0.03)	1.01 (±0.01)	0.99 (±0.01)	1.01 (±0.01)	1.00 (±0.02)	1.00 (±0.02)	1.00 (±0.01)
P–D,L-Y	0.99 (±0.02)	1.01 (±0.02)	1.00 (±0.02)	0.99 (±0.01)	1.00 (±0.01)	1.01 (±0.01)	1.01 (±0.02)
P–O (No template)	1.01 (±0.01)	1.01 (±0.03)	1.01 (±0.01)	0.99 (±0.01)	1.01 (±0.02)	1.01 (±0.03)	0.99 (±0.01)

In a typical experiment, 2 g of the polymer (total Cu capacity ~50 μmol) was equilibrated with a 5 m*M* amino acid solution at pH 9–9.5 (2-fold excess). The polymer code denotes the amino acid template using the one letter code. P–O is the control material synthesized in the absence of template.

[a] $\alpha_{L/D}$ is reported for polymers imprinted with L-amino acids, and $\alpha_{D/L}$ for those imprinted with D-amino acids. $\alpha_{L/D}$ is reported for the control materials. Selectivities of ~1.1 or higher are indicated in italics. Error limits are based on three measurements of optical rotation and five measurements of absorbance for each sample. The numbers reported here are averaged over three separate trials of each experiment.

[b] Enantioselectivity for tryptophan was tested only for P–O and the polymers imprinted with phenylalanine and tyrosine

corresponding imprinted polymers show significant cross-selectivity. For amino acids with smaller or more flexible side chains as compared to the template, there is little enantioselectivity. When the side chain is larger than that of the template amino acid, the enantioselectivity also decreases (cf. tryptophan binding to phenylalanine-imprinted polymers and phenylalanine binding to leucine-imprinted polymers). It is also possible that π–π interactions between the styryl group of the VBIDA monomer and the amino acid side group are contributing to the binding of the aromatic amino acids and to the observed selectivities.

3.4. Column chromatography

Enantioresolution of racemic phenylalanine was studied by column chromatography. For this purpose, 10-μm diameter macroporous silica beads (pore size ~1000 Å) were coated with polymeric material imprinted for phenylalanine and slurry-packed in an HPLC column (50 mm×4.6 mm I.D.). The material was packed in the metal-ion free form and copper ions were reloaded under flow. It was not possible to achieve efficient resolution using an acetate buffer as a competitor in the eluent; the phenylalanine was strongly bound due to metal chelation and was

retained for more than 10 column volumes. When acetate was replaced by 1.5 m*M* glycine, the retention volumes were reduced and more efficient separations were obtained. Fig. 4a shows the separation of D,L-phenylalanine on the adsorbent imprinted with D-phenylalanine. The separation factor is 1.65. The template enantiomer is more strongly retained, eluting as a broad band between 5 and 8 column volumes, while the opposite enantiomer eluted between 3 and 4 column volumes. Similar results were obtained for the support prepared using L-phenylalanine as the template. The control material prepared in the absence of any template was similarly tested in the Cu(II) reloaded form. On this column, D,L-phenylalanine eluted as a single peak between 3 and 4 column volumes, comparable to L-phenylalanine on a material imprinted with D-phenylalanine and vice versa.

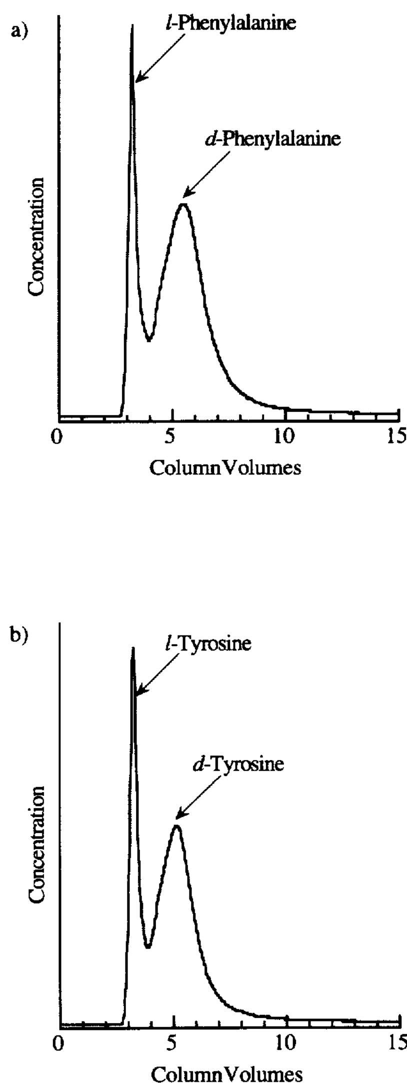


Fig. 4. Chromatographic resolution of (a) D,L-phenylalanine and (b) D,L-tyrosine on D-phenylalanine-imprinted polymer-coated silica (50×4.6 mm I.D. column). Peak identification was confirmed by comparison with the retention times of the pure enantiomers. Sample size: 100 μl of 1 m*M* solution. Running conditions: 1 ml min^{-1}, 50°C, 1.5 m*M* glycine. Chromatographic separation factors are (a) 1.65 for D,L-phenylalanine and (b) 1.54 for D,L-tyrosine.

The phenylalanine-imprinted supports were also able to resolve D,L-tyrosine into its enantiomers, with a separation factor similar to that observed for D,L-phenylalanine ($\alpha = 1.54$) (Fig. 4b). Racemic tryptophan eluted as a broad band between 2 and 5 column volumes, but was not resolved on this column under these conditions (data not shown). These columns were also unable to resolve racemic mixtures of alanine, valine, leucine or isoleucine. Finally, samples containing equimolar amounts of phenylalanine and tyrosine enantiomers of like chirality also eluted as single peaks on the column imprinted with pure enantiomers of phenylalanine. While the molecularly-imprinted materials can select for the chirality of the template, they are unable to recognize the small size difference between phenylalanine and tyrosine. The inability of these imprinted materials to recognize small variations of the amino acid side chain is also consistent with the equilibrium rebinding observation that polymers imprinted with alanine showed no measurable enantioselectivity.

These results are consistent with the hypothesis that the size of the template amino acid plays an important role in imparting chiral selectivity. Material obtained by imprinting with a phenylalanine enantiomer is specific only for phenylalanine and closely-related tyrosine. Alanine and valine have smaller side groups compared to phenylalanine, and both enantiomers of these amino acids presumably can fit into binding sites that will distinguish phenylalanine enantiomers. Thus they are retained, but not enantioselectively. The lack of enantioselectivity for leucine and isoleucine exhibited by the phenylalanine-imprinted materials is probably due to the flex-

ibility of the aliphatic side groups. The retention of tryptophan is slightly greater than for the other amino acids, on the control material prepared in the absence of template amino acid as well as on the imprinted materials, possibly due to additional interactions of the indole side chain with the immobilized copper ions. The larger size of the indole side group could mean that both enantiomers of tryptophan face comparable steric hindrance to binding in a polymer imprinted for a phenylalanine enantiomer. Therefore we see reduced enantioselectivity for tryptophan in the equilibrium rebinding experiment and no enantioresolution in the chromatographic mode.

3.5. Mechanisms of enantioselectivity

Possible sources of enantioselectivity observed in the molecularly-imprinted ligand-exchange polymers will be discussed in the light of the 'three-point interaction' model for conventional CSPs used in LEC. This model requires at least three points of interaction between the chiral selector and the targeted enantiomer for chiral recognition and separation [5,14]. In LEC of amino acids on an L-proline bonded phase or with a L-proline metal complex in the mobile phase, at least one point of interaction must be between the mixed-metal:ligand complex and the solid support. In conventional LEC supports, at least two points of interaction are available for both enantiomers: simultaneous coordination of the amine and carboxylate groups to the metal ion. The good selector that discriminates between the two enantiomers should therefore stabilize the third interaction with one of the enantiomers to the maximum possible extent. Chiral selectivity can also be achieved if the third interaction is destabilized for one of the enantiomers relative to the other. The nature of the interaction of the mixed ligand complex with the support matrix thus plays a crucial role in chiral recognition, and this interaction can vary significantly, even for closely-related materials.

It is not immediately clear where the origins of chiral selectivity lie for the molecularly-imprinted ligand-exchange materials. Because we have used an achiral crosslinker and an achiral metal complex attached to the support, the interaction that decides the enantioselectivity must arise either from the residual incorporation of the chiral template amino acid or from the formation of enantioselective binding sites in the polymers imprinted with chiral template. We do not believe that residual incorporation of chiral template can explain our results. This conclusion is based on the small amount of template remaining in the polymers after washing, the nature of the coordination/chelation interaction, and the fact that the enantioselectivities obtained by molecular imprinting increase with the size of the amino acid side chain. The polymers contain less than 0.8 $\mu mol\ g^{-1}$ residual template, as compared to ~25 $\mu mol\ g^{-1}$ for the amount of template and metal ions that can be reversibly bound. The metal ions and template that could not be removed by washing with EDTA are probably inaccessible to both EDTA and any more amino acid. In any case, the residual template cannot account for the selectivities measured, even if those few sites were infinitely selective for rebinding substrate of like chirality and the remaining sites had zero selectivity. For example, the equilibrium rebinding experiments utilized a two-fold excess of racemic substrate. The maximum enantioselectivities that could be obtained by this route are only ~13/12=1.08, considerably less than those observed for the larger amino acids. In addition, the specific directional nature of the metal coordination/chelation bonds would make it difficult for a second amino acid to bind to a metal ion already bound to iminodiacetate and a template molecule. Finally, although the levels of residual amino acid incorporated in the imprinted polymers are similar, the enantioselectivities of these materials are markedly different.

The remaining explanation is that molecular imprinting achieves chiral recognition through the formation of chirally-selective binding sites. In light of the three-point interaction model, this implies that formation of the polymer network around the monomer–template complex stabilizes the binding of the template enantiomer and/or destabilizes (sterically hinders) binding of the opposite enantiomer. As illustrated in Fig. 5 for a polymer templated with D-phenylalanine, rebinding of the D-enantiomer proceeds through chelation of the metal ion, in addition to which the aromatic side chain fits into a cavity that selects for both the size and shape of this group. In contrast, metal chelation by the L-enantio-

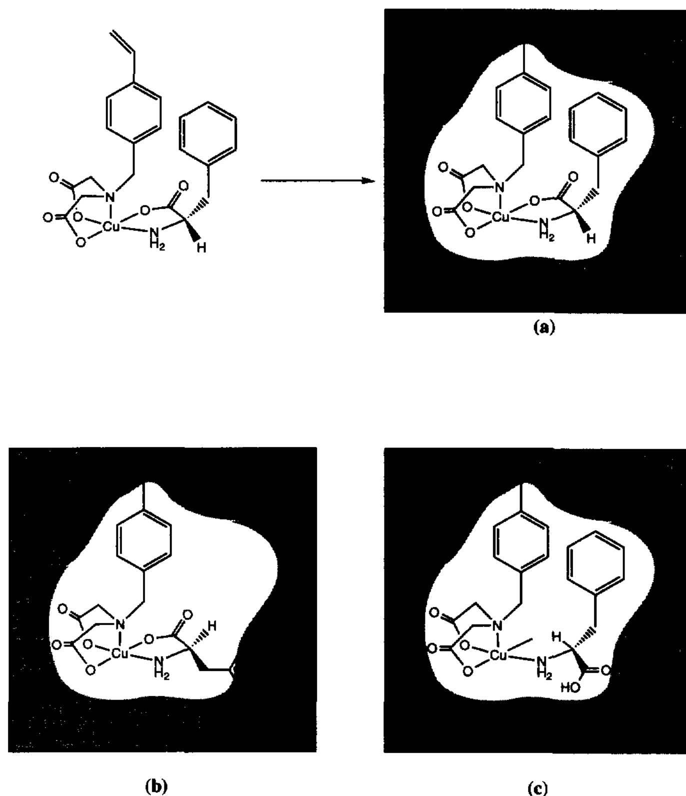


Fig. 5. Source of enantioselectivity in imprinted ligand-exchange materials. Molecular imprinting with L-phe gives a cavity that is selective for L-phe. (a) The L-isomer can simultaneously chelate to metal ion and fit into the shape-selective cavity. (b) Rebinding of the D-isomer is hindered because chelation of the metal ion by the D-isomer is sterically unfavorable. (c) Alternately, if the molecule fits into the cavity, it cannot chelate Cu(II). This is idealized picture of the origin of enantioselectivity is probably true only for a small fraction of the binding sites.

mer would be sterically hindered. Alternatively, if the side group of the L-amino acid fits into the cavity, only monodentate binding to the metal would be possible. Either one would destabilize the metal chelation interaction. If this were the only mechanism for enantioselectivity, the magnitude of this destabilization could be estimated from the magnitude of the selectivity. The maximum separation factor seen on any of these imprinted materials is 1.65 in the case of phenylalanine, which corresponds to a free energy difference of only ~300 cal mol^{-1} (1 cal=4.184 J). The energetics of the metal chelation interaction compared to the weak steric destabilization imposed by the cavity.

The above explanation is based upon an idealized view of the nature of the binding cavities obtained by molecular imprinting. The maximum separation factor obtained for phenylalanine enantiomers is comparable to that shown by the conventional ligand-exchange CSPs [5]. However, baseline separations have been obtained on the conventional CSPs, whereas there is a large degree of peak overlap in the chromatograms from molecularly imprinted materials. The poor resolution is accompanied by greater broadening of the peak corresponding to the more strongly retained enantiomer. These features, which have been observed in separations on other molecularly imprinted materials, have been generally attributed to heterogeneity in the binding sites [12,13,16]. Molecular imprinting seems to result in the formation of binding sites with a distribution of binding strengths. Fig. 5 thus probably illustrates only the

most selective sites. An average over all binding sites is measured in a separation experiment.

The above explanation of the origin of enantioselectivity in these imprinted materials is applicable to amino acids, which chelate the metal ion. However, in the case of chiral molecules that bind to metal ions in a monodentate fashion, the metal coordination interaction will provide only one of the three necessary for enantioselectivity. Thus, in order to successfully resolve molecules such as chiral amines or carboxylic acids, molecular imprinting has to contribute the equivalent of at least two other points of interaction between the polymer matrix and the substrate. To further probe the nature of the enantioselectivity of the molecularly-imprinted adsorbent, we tested the imprinted polymer-coated silica column synthesized for phenylalanine for its ability to separate two racemates which are analogous to phenylalanine and which coordinate to the metal ion only in a monodentate fashion, α-methylphenethylamine and α-methylhydrocinnamic acid (Fig. 6). Glycine binds more tightly to the metal ion than α-methylphenethylamine and α-methylhydrocinnamic acid, causing both to elute at close to one column volume without enantioresolution. Thus the chromatography was carried out using 1.5 m*M* acetate at pH 8 instead of glycine in the elution buffer. The column imprinted with D-phenylalanine was able to resolve the chiral amine, as shown in Fig. 7. However, the chiral carboxylic acid still eluted early without being resolved. Further attempts to resolve the chiral carboxylic acid by reducing the pH were unsuccessful.

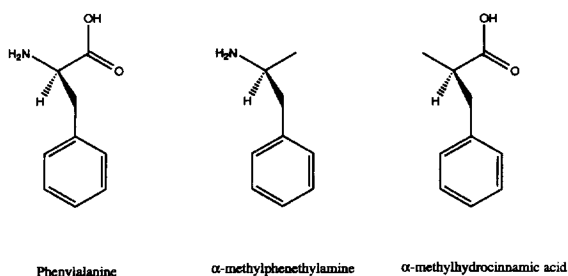


Fig. 6. Phenylalanine analogs α-methylphenethylamine and α-methylhydrocinnamic acid.

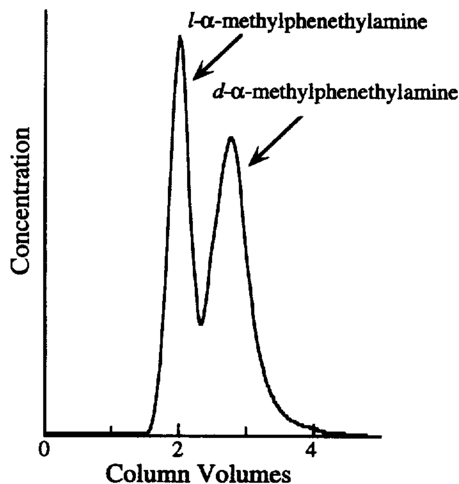


Fig. 7. Chromatographic resolution of α-methylphenethylamine on column packed with phenylalanine-imprinted polymer-coated silica (50 mm×4.6 mm I.D.). Sample size: 100 μl of 1 m*M* solution. Running conditions: 1 ml min^{-1}, 50°C, 1.5 m*M* acetate, pH 8. Chromatographic separation factor is 1.32.

Although the chiral amine cannot chelate metal ions, the polymer imprinted with a phenylalanine enantiomer was able to resolve its chiral amine analog. The chiral amine binds more strongly than acetate to the metal complex. Therefore in the presence of competition from acetate, the amine is retained; it is also resolved into its enantiomers. However, binding of the carboxylic acid analog is not strong enough to result in significant retention in the presence of competition from acetate. Seen in the light of the three-point interaction model, the ability of the material imprinted with a phenylalanine enantiomer to resolve an analogous chiral amine indicates that the steric interaction between the side group and the binding cavity involves more than one point of contact. (π–π interactions between the functional monomer and the amino acid side chain might also contribute a point of interaction.) Thus there is sufficient evidence here to conclude that molecular imprinting with a chiral template has created chirally-selective binding cavities. This selectivity must result from a microstructure of the binding cavity that is complementary to the side group of the template amino acid.

4. Conclusions

This is the first report of the preparation of molecularly-imprinted polymers for the chiral separation of underivatized amino acids. The same polymers can be used to resolve related chiral amines. The amino acid template forms the stronger complex, which is advantageous for the fidelity of molecular imprinting [17]. Both size and shape of the amino acid side group contribute to the chiral resolution. The use of achiral reactants has highlighted the role of molecular imprinting in creating the enantioselective adsorbents. It may be possible to improve the enantioselectivities of these materials further by choosing appropriate chiral functional monomers. Alternatively, the use of comonomers or crosslinkers that provide additional interactions with the side groups of the amino acid templates could contribute to improved resolution, provided the stabilization is preferential for the targeted enantiomer.

Acknowledgments

This research is supported by the National Science Foundation (BES-9416915).

References

[1] S.V. Rogozhin, V.A. Davankov, J. Chem. Soc. D (1971) 490.
[2] H. Caruel, L. Rigal, A. Gaset, J. Chromatogr. 558 (1991) 89–104.
[3] G. Gubitz, B. Pierer, W. Wendelin, Chirality 1 (1992) 333–337.
[4] B. Lefebvre, R. Audebert, C. Quivoron, J. Liq. Chromatogr. 1 (1978) 761–770.
[5] V.A. Davankov, in D. Caignant (Editor), Complexation Chromatography (Chromatographic Science Series, Vol. 57), Marcel Dekker, New York, 1992, pp. 197-245.
[6] K. Mosbach, Trends Biochem. Sci. 19 (1994) 9–14.
[7] G. Wulff, Angew. Chem. 34 (1995) 1812–1832.
[8] S. Vidyasankar, F.H. Arnold, Current Opinion Biotechnol. 6 (1995) 218–224.
[9] P.K. Dhal, F.H. Arnold, Macromolecules 25 (1992) 7051–7059.
[10] B. Sellergren, M. Lepisto, K. Mosbach, J. Am. Chem. Soc. 110 (1988) 5853–5860.
[11] B. Sellergren, G. Ekberg, K. Mosbach, J. Chromatogr. 347 (1985) 1–10.
[12] L. Andersson, B. Sellergren, K. Mosbach, Tetrahedron Lett. 25 (1984) 5211–5214.
[13] M. Kempe, K. Mosbach, Anal. Lett. 24 (1991) 1137–1145.
[14] V.A. Davankov, A.A. Kurganov, Chromatographia 17 (1983) 686–690.
[15] Y. Fujii, K. Matsutani, K. Kikuchi, J. Chem. Soc., Chem. Commun., (1985) 415–417.
[16] S.D. Plunkett, F.H. Arnold, J. Chromatogr. A 708 (1995) 19–29.
[17] F.H. Arnold, S.D. Plunkett, S. Vidyasankar, Polymer Prepr. 35 (1994) 996–997.
[18] A.K. Rao, G.N. Kumar, M.S. Mohan, Y.L. Kumari, Ind. J. Chem. A 31 (1992) 256–259.
[19] A.E. Martell, R.M. Smith, Critical Stability Constants, Plenum Press, New York, 1974.