Anal. Chem. **1998,** *70,* 2789−2795

Molecularly Imprinted Polymeric Adsorbents for Byproduct Removal

Lei Ye,* Olof Ramström, and Klaus Mosbach

Pure and Applied Biochemistry, Chemical Center, Lund University, P.O. Box 124, S-221 00 Lund, Sweden

In this study, both diastereo- and enantioselective adsorbents for a dipeptide derivative were prepared using a molecular imprinting technique. The diastereo- and enantioisomers for the dipeptide derivative *N*-(benzyloxycarbonyl)aspartylphenylalanine methyl ester (ZAPM), in addition to the α- and *β*-isomers, were chosen as test compounds for the investigation of the imprinting effect. The close similarities between the structures of different isomers make it possible to interpret the roles of template structure on specific molecular recognition. A highly specific byproduct scavenger was prepared by simultaneously incorporating methacrylic acid and vinylpyridine as functional monomers. The binding selectivities of polymeric adsorbents for the α- and *β*-isomers are shown to be greatly enhanced by introducing enantiocomplementarities into the polymer matrixes. An anti-*β*-L,L-ZAPM polymer was applied in a solid-phase extraction protocol, for the purification of the product in the chemical synthesis of N-protected aspartame. Finally, polymer beads were also imprinted against *β*-L,L-ZAPM using suspension polymerization performed in perfluorocarbon fluid. The imprinted polymer beads displayed the same binding characteristics as the imprinted bulk polymer and can be envisaged for the use of product purification in chromatographic mode.

Analogous to natural recognition units such as enzymes, antibodies, and receptors, various synthetic mimics of these bioactive components have been produced by molecular imprinting techniques.[1−4] The print molecule can be removed after the microscopic molding process and the obtained polymeric matrixes become ready to recognize the corresponding print species. The diastereo- and enantiospecificities of molecularly imprinted polymers much depend on the complexation between the print molecule and the functional monomers during polymerization, as well as between the analyte and the prearranged polymer functionalities in the later binding process. In the noncovalent strategy, complex formation (self-assembling) is based on the noncovalent interactions between the print molecule and the functional monomers. Among the most often employed are hydrogen bonds and ionic interactions. So far, since most noncovalently imprinted polymers have been prepared in less polar organic solvents, hydrophobic interactions have not been extensively utilized, at least not as a main interaction for polymer preparation. However, these may, in some cases, enhance the recognition capabilities for the obtained polymers when analyzed in an aqueous environment. From a practical point of view, the easy self-assembling in polymer preparation and the fast recognition kinetics of the obtained polymers have made the noncovalent strategy attractive for applications in different areas. Noncovalently imprinted polymers have been explored as tailor-made chiral separation phases,[5] molecularly imprinted "immuno" assays,[6,7] recognition units in sensors,[8] and solid-phase extraction materials.[9] Spherical, molecularly imprinted polymer beads exhibited better flow characteristics in chromatography and higher binding capacities compared to ordinary bulk polymers, which are ground and sieved to provide irregularly shaped fragments.[10,11] The molecularly imprinted polymer beads may extend molecular imprinting to more practical applications. In addition, we have demonstrated a novel application of molecularly imprinted polymers (MIPs) as auxiliary reagents in enzymatic reactions, e.g., the synthesis of an artificial sweetener precursor, Z-aspartame.[12]

For the noncovalent preparation of MIPs, the print molecule forms complexes with one or more molecules of functional monomers. While a single functional monomer, of either acidic or basic nature, is used routinely, simultaneously utilizing multiple functional monomers provides more possibilities in achieving the optimal complexation between the print molecule and the monomers and, therefore, may enhance the recognition capabilities. It has been found that the recognition capabilities of MIPs for derivatized amino acids can be improved by introducing two chemically distinct functional monomers.[13] In the present study, this approach is extended to a dipeptide derivative, *N*-(benzyloxycarbonyl)aspartylphenylalanine methyl ester (ZAPM). Considering the versatile isomers of this dipeptide family and the close similarities among them, the effects of both template structure

* To whom correspondence should be addressed: (phone) +46 46 2229560; (fax) +46 46 2224611; (e-mail) Lei.Ye@tbiokem.lth.se.

(1) Mosbach, K.; Ramström, O. *Bio/Technology* **1996**, *14*, 163−170.
(2) Ansell, R. J.; Kriz, D.; Mosbach, K. *Curr. Op. Biotechnol.* **1996**, *7*, 89−94.
(3) Shea, K. J. *Trends Polym. Sci.* **1994**, *2*, 166−173.
(4) Wulff, G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1812−1832.
(5) Kempe, M. *Anal. Chem.* **1996**, *68*, 1948−1953.
(6) Vlatakis, G.; Andersson, L. I.; Müller, R.; Mosbach, K. *Nature* **1993**, *361*, 645−647.
(7) Ramström, O.; Ye, L.; Mosbach, K. *Chem. Biol.* **1996**, *3*, 471−477.
(8) Kriz, D.; Ramström, O.; Svensson, A.; Mosbach, K. *Anal. Chem.* **1995**, *67*, 2142−2144.
(9) Muldoon, M. T.; Stanker, L. H. *Anal. Chem.* **1997**, *69*, 803−808.
(10) Mayes, A. G.; Mosbach K. *Anal. Chem.* **1996**, *68*, 3769−3774.
(11) Ansell R. J.; Mosbach K. *J. Chromatogr., A* **1997**, *787*, 55−66.
(12) Ramström, O.; Ye, L.; Krook, M.; Mosbach, K. *Chromatographia* **1998**, *47*, 465−469.
(13) Ramström, O.; Andersson, L. I.; Mosbach, K. *J. Org. Chem.* **1993**, *58*, 7562−7564.

S0003-2700(98)00069-9 CCC: $15.00
Published on Web 05/28/1998

and the acidities of functional groups on imprinting and recognition can be investigated. Separation of different stereoisomers in this dipeptide derivative family is practically beneficial. Among all the isomers, only *N*-(benzyloxycarbonyl)-α-L-aspartyl-L-phenylalanine methyl ester (α-L,L-ZAPM) is a useful precursor for the artificial sweetener; the byproduct, e.g., *N*-(benzyloxycarbonyl)-*β*-L-aspartyl-L-phenylalanine methyl ester (*β*-L,L-ZAPM), that may form from the chemical synthesis has to be removed. As imprinted polymers persist in both diastereo- and enantioselective memory for their print molecules, their potential applications as polishing agents in downstream purification can also be envisaged. We present here a molecularly imprinted scavenger for byproduct removal in the chemical synthesis of aspartame. Molecularly imprinted polymer beads were also prepared using an optimized recipe aiming at the typical target; the beaded MIP displayed the same binding characteristics of the bulk MIP and can be used as a stationary phase for chromatographic product purification.

EXPERIMENTAL SECTION

Materials. Z-L-Asp-L-Phe-OMe (α-L,L-ZAPM) was purchased from Indofine Chemical Co. (Somerville, NJ). Z-D-Asp-D-Phe-OMe (*β*-L,L-ZAPM) was from Eurodiagnostica (Malmo, Sweden). Z-L-aspartic acid (Z-L-Asp), Z-D-aspartic acid (Z-D-Asp), L-phenylalanine methyl ester hydrochloride (L-Phe-OMe·HCl), and D-phenylalanine methyl ester hydrochloride (D-Phe-OMe·HCl) were from Sigma (St. Louis, MO). Perfluoro(1,3-dimethylcyclohexane) (PMC) was from Fluorochem (Old Glossop, U.K.). Other chemicals were from commercial sources. All solvents were of either analytical or HPLC grade. L-Phe-OMe and D-Phe-OMe were prepared by treatment of respective hydrochloride salt with equimolar sodium carbonate, *β*-L,L-ZAPM, *β*-D,D-ZAPM, *β*-L,D-ZAPM and *β*-D,L-ZAPM were prepared by coupling corresponding Z-aspartic anhydride with phenylalanine methyl ester in dimethyl sulfoxide.[14] Optical activities ($[\alpha]^{20}_{589}$) of the synthesized dipeptides in methanol were confirmed with an AA-1000 polarimeter (Optical Activity Ltd, England): *β*-L,L-ZAPM, +10.0; *β*-D,D-ZAPM, −10.0; *β*-L,D-ZAPM, −4.6; *β*-D,L-ZAPM, +4.6.

Preparation of Bulk MIPs. Molecularly imprinted bulk polymers were prepared according to Table 1. In a typical preparation, 1 mmol of print molecule, a stated amount of functional monomers, 40 mmol of EDMA, and 100 mg of 2,2′-azobis(2,4-dimethylvaleronitrile) (ABDV) were dissolved in 12 mL of acetonitrile, and the polymerization solution was subsequently purged with N_2 for 5 min, while cooled in an ice bath. Polymerization was thermally induced at 45 °C and proceeded for 16 h. The resulting bulk polymer monolith was ground in a mechanical mortar and wet-sieved. Particles with diameter smaller than 25 μm were collected from which fine particles were removed by repeated sedimentation in acetone. The blank polymer, B-1, was prepared in the same way except that no print molecule was used.

Preparation of Beaded MIPs. Molecularly imprinted polymer beads were prepared using suspension polymerization performed in a perfluorocarbon fluid, as described previously.[10,11] Synthesis of the perfluorinated polymeric surfactant (PFPSA) was described elsewhere.[11] Fifty milligrams of PFPSA was dissolved in 0.5 mL of 1,2-dichloroethane (DCE) and then mixed with 20 mL of PMC to form the dispersing phase. The imprinting mixture, which was composed of 125 μmol of *β*-L,L-ZAPM, 1 mmol 4-vinylpyridine (4Vpy), 2 mmol of methacrylic acid (MAA), 4 mmol of trimethylolpropane trimethacrylate (TRIM), 20 mg of ABDV, and 3.5 mL of DCE, was then added and emulsified by stirring at 2000 rpm for 5 min. The emulsion was purged with nitrogen for 5 min and then polymerized at 55 °C for 16 h; the final beaded polymer product was obtained after workup. The polymer beads were packed into a 100 × 4.6 mm stainless steel HPLC column; the print molecule was washed away using methanol containing 10% acetic acid as a mobile phase, until a stable baseline was obtained.

Table 1. Preparation and Chromatographic Characterization of Molecularly Imprinted Polymers[a]

polymer	print molecule	composn of functional monomer (mmol) 4VPy/MAA	*k′* L,L	*k′* D,D	α	f/g	extrn effect α/*β*
α-1	α-L,L-ZAPM	8/16	2.2	1.2	1.9	0.5	3.2
α-2	α-L,L-ZAPM	8/8	1.5	1.0	1.5	0.5	3.3
α-3	α-L,L-ZAPM	8/0	0.8	0.7	1	0	2.5
β-1	*β*-L,L-ZAPM	8/16	3.0	2.1	1	0	4.2
β-2	*β*-L,L-ZAPM	8/8	4.1	1.8	2.5	0.9	3.7
β-3	*β*-L,L-ZAPM	8/0	1.6	1.2	1.3	0.5	2.4
B-1		8/16	0.4[b]	0.4[b]	1[b]	0[b]	3.4
			0.7[c]	0.7[c]	1[c]	0[c]	
B-2	*β*-D,D-ZAPM	8/16	nd[d]	nd	nd	nd	nd

[a] For the preparation of imprinted polymers, 1 mmol of print molecule, 40 mmol of EDMA, and 100 mg of ABDV were used. For the chromatographic analysis of MIPs, polymer particles were packed into a 100 × 4.6 mm HPLC column. Acetonitrile containing 1% acetic acid was used as the mobile phase; acetone was used as the void marker. Capacity factor *k′* was determined by separately injecting 8.5 μg of print molecule or its enantiomer. Separation factors, α, and f/g values were calculated from injecting 8.5 μg of racemate mixture. For the determination of the extraction effect, 7 μmol of α-L,L-ZAPM and 3 μmol of *β*-L,L-ZAPM (α/*β* = 2.3, mol/mol) were incubated with 150 mg of polymer in acetonitrile at room temperature for 1 h. The molar ratio of the two isomers in supernatant was determined by reversed-phase HPLC analysis. [b] α-Isomer was analyzed. [c] *β*-Isomer was analyzed. [d] not determined.

Chromatographic Analysis of Molecularly Imprinted Polymers. Polymer particles were suspended in 40 mL of chloroform and packed into a standard 100 × 4.6 mm stainless steel HPLC column, using acetone as packing solvent at 300 bar, with an air-driven fluid pump (Haskel Engineering Supply Co., Burbank, CA). Chromatographic analyses were performed isocratically using a Pharmacia LKB type 2249 solvent delivery system and a variable-wavelength monitor model 2141 (Pharmacia LKB Biotechnology), together with a software package EZChrom (Scientific Software, CA). Acetonitrile containing 1−2% acetic acid (v/v) was used as mobile phase at a flow rate of 0.5 mL/min and the analytes were monitored at 254 nm. Acetone was used as the void marker. The capacity factor (*k′*) and separation factor (α) were calculated using basic chromatography theory. The f/g value (between 0 and 1) was determined according to Meyer,[15] with a f/g of 1 represent baseline separation. For the estimation of *k′*, 8.5 μg of single analyte was injected. Separation factors and f/g values were calculated from the chromatogram obtained by applying 8.5 μg of mixed analytes (at equal amounts for each).

Chemical Synthesis of N-Protected Aspartame. Z-L-Aspartic anhydride (2 mmol) was dissolved in 20 mL of acetonitrile;

(14) Yang, C. P.; Su, C. S. *J. Org. Chem.* **1986**, *51*, 5186−5191.

(15) Meyer, V. R. *Chromatographia* **1987**, *24*, 639−645.

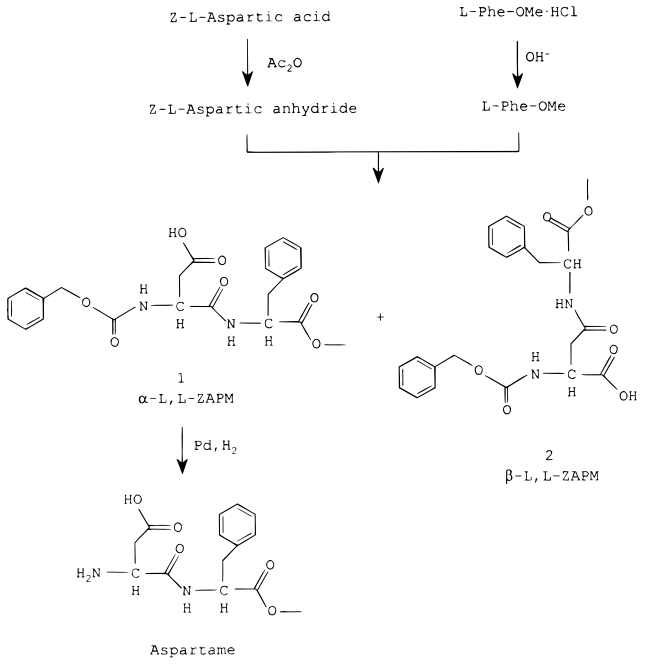

Figure 1. Chemical synthesis of aspartame.

2 mmol of L-phenylalanine methyl ester was then added with stirring. The reaction proceeded at room temperature for 2 h. The composition of the coarse product was determined by reversed-phase HPLC analysis.

Solid Phase Extraction Using Molecularly Imprinted Polymers. Sample solutions simulating the chemical synthetic output was prepared by mixing specific amounts of α-L,L-ZAPM and β-L,L-ZAPM in the corresponding solvent. Polymers were applied with a dosage of 150 mg/mL sample solution. Extraction was performed by gently mixing the suspension at room temperature for 1 h. After centrifugation, the composition of the supernatant was analyzed by reversed-phase HPLC. For successive extraction, the supernatant from the previous extraction was mixed with the same amount of new adsorbent, and extraction was then performed under the same conditions. Solutions of the chemically synthesized coarse products were diluted 10 times with the reaction solvent before it was used for extraction experiment.

Reversed-Phase HPLC Analysis. Reversed-phase HPLC analysis was performed on the same system as that used for polymer characterization. An ODS column (Nucleosil 100-5C18) was used with a mobile phase composed of equal volumes of water and 10 mM NaH_2PO_4 buffer solution (pH 4.15), with a flow rate of 0.5 mL/min. Sample solution (200 μL) was dried under vacuum, the residue was then dissolved in 2 mL of mobile phase, 20 μL of this solution was injected, and eluent was monitored at 210 nm.

RESULTS AND DISCUSSION

Preparation of Molecularly Imprinted Bulk Polymers. Two isomers, α-L,L-ZAPM and β-L,L-ZAPM were used as print molecules. The only differences between them are the position of the free carboxyl group (Figure 1) and, therefore, their relative acidities and the distances between the acidic functionalities and the first chiral center, which were supposed to play important roles in complex formation. The traditional, widely used acidic and basic functional monomers, MAA and 4VPy, respectively, were used in this investigation. MIPs using only one kind of functional monomer, as well as simultaneously using both, were prepared. Ethylene glycol dimethacrylate (EDMA) has been utilized successfully in various protocols and is well documented as an ideal cross-linker for noncovalent imprinting. The relatively low viscosity of this cross-linker makes it advantageous in homogenizing the prepolymerization mixture. Another methacrylate-related cross-linker, TRIM, which has one more polymerizable vinyl unit than EDMA, has also been used previously. It has been shown that polymers prepared using TRIM as cross-linker were partially macroporous, and when these MIPs were used for chromatographic separation, they provided superior separation factors and loading capacities.[5] In this study, EDMA was exclusively used for the preparation of bulk MIPs, and TRIM was used later for the preparation of beaded MIP. All polymers were prepared by heat-induced polymerization using ABDV as an initiator. The binding specificities of the obtained polymers were then characterized in chromatographic mode. Ideally, the imprinting effect can be evaluated by analyzing the enantioselectivities of the obtained polymers. On the other hand, by choosing a proper reference compound/polymer, other ligand selectivities can also provide valuable information regarding the outcome of an imprinting process.

Template Structure and Enantioselectivities of Imprinted Bulk Polymers. In molecular imprinting, it is important to comprehend the working roles of the submolecular units, e.g., certain functional groups on the print molecule, for the complexation with the polymerizable, functional monomers during polymerization, as well as with the prearranged, template-directed distribution of polymeric functionalities in the later binding process. The former was previously addressed, with the proposal of a model of 1:2 complex between phenylalanine anilide, the print molecule, and methacrylic acid.[16] So far no effort has been made for the latter, which may be addressable with the use of a solid-state NMR technique. Instead of the direct investigation, some information regarding the above-mentioned issues could be envisaged in an indirect way. For the typical dipeptide derivative, Z-Asp-Phe-OMe, there are several different isomers, including both diastereo- and enantioisomers. The close similarities among these structures make them appropriate candidates in investigating factors that affect complexation and recognition.

When only methacrylic acid was used as a functional monomer for the preparation of a bulk polymer imprinted against α-L,L-ZAPM (structure 1, Figure 1), and with a molar ratio of α-L,L-ZAPM:MAA:EDMA = 1:10:50, the obtained polymer gave a larger capacity factor (k') for the print molecule than for its enantiomer, α-D,D-ZAPM, when administered separately. While the difference between these capacity factors confirms an imprinting effect, separation of 8.5 μg of racemate on a 100 × 4.6 mm analytical HPLC column packed with the polymer was, however, not successful (elution condition was not optimized, data not shown). Likewise, no separation was recorded of a racemic sample when applied to polymer α-3, which was prepared using only 4Vpy as a basic functional monomer. When both MAA and 4Vpy were introduced, it was found that the imprinted polymer did produce

(16) Sellergren, B.; Lepistö, M.; Mosbach, K. *J. Am. Chem. Soc.* **1988**, *110*, 5853−5860.

separation of the racemate under the same conditions (Table 1). The separation factor increased when the second basic functional monomer was introduced, with a molar ratio of 4VPy to MAA of 1:2. Further increase of 4VPy, however, produced less separation, as characterized by decreased α and f/g values. As there is a free carboxyl group on the side chain of the print molecule, ionic interactions could be introduced by employing a basic monomer, e.g., 4VPy, which becomes partly protonated under these conditions. Utilization of the stronger ionic interaction makes polymer α-1 and α-2 better enantioselectors, compared with the polymer prepared using only an acidic functional monomer. The fact that polymer α-3 gives no racemic separation may probably be because vinylpyridine is a poor hydrogen-bonding monomer. The function of methacrylic acid in preparing the imprinted poly(MAA−4VPy−EDMA) might be double: it increases the protonation of 4-vinylpyridine and thus enhances the interactions between the print molecule and functional monomers; it also provides hydrogen bond interactions with the template. One thing that needs to be mentioned is that methacrylic acid can also interact ionically with the basic monomer, which is detrimental for imprinting. The success of imprinting depends much on appropriately adjusting all the reaction components.

The free carboxylic group in β-L,L-ZAPM "sits" on the main chain and has a lower pK_a value than α-L,L-ZAPM.[17] The β-L,L-ZAPM-imprinted polymers have binding characteristics different from that of the polymers imprinted against the α-isomer. While the imprinted poly(MAA−EDMA) gives no separation of the racemic mixture, the poly(4VPy−EDMA) produces a capacity factor of 1.3 and a f/g value of 0.5. It may be proposed that the vinylpyridine monomer could be effectively protonated by, and interact with, the carboxylic group in the print molecule. The polymer prepared by using a molar ratio of β-L,L-ZAPM:4VPy:MAA:EDMA = 1:8:8:40 shows the best separation, with a capacity factor of 2.5 and a f/g value of 0.9. Further addition of MAA makes the obtained polymer less efficient in racemic separation. In this later case, the by-effect of MAA as an interfering factor in the MAA−4VPy−EDMA tertiary system may dominate. What can be inferred from these results is that the relative amount of acidic and basic functional monomers in the tertiary acidic monomer−basic monomer−cross-linker system should be optimized according to the acidity or basicity of the print molecule in order to make a good imprint. The more acidic the print molecule is, the more basic monomer will need to be used, and vice versa.

On the columns packed with bulk anti-β-L,L-ZAPM MIPs, capacity factors of the β-related ligands have a decreasing order as β-L,L > β-L,D > β-D,L > β-D,D (Table 1, Table 2). It can be seen that the capacity factor of the ligand increases with its similarity to the print molecule. What can also be proposed is that the main-chain carboxylic group is important in both imprinting and the later recognition process.

Enhancement of Binding Specificities by Molecular Imprinting. Some recent applications of MIPs were found in the area of solid-phase extraction (SPE), as originally proposed by Sellergren.[18−20] Similar to ordinary polymeric adsorbents, the nonimprinted poly(MAA−EDMA) could actually display some nonspecific extraction effects, which make the proper choice of reference a great challenge. For the rational design of a molecularly imprinted polymer for solid-phase extraction, one has to be sure that in addition to the nonspecific binding, the adsorption comes, at least partially, from the specific interaction between ligand and the MIP. However, this has not yet been verified satisfactorily by previous investigations. In the present study, we are interested in the selective adsorption of β-L,L-ZAPM, a byproduct in the chemical synthesis of α-L,L-ZAPM (Z-aspartame, a synthetic precursor for an artificial sweetener). The molecularly imprinted adsorbents could be used as a polishing agent in downstream processing, for the purification of the target product. To investigate the binding specificities, bulk polymers imprinted against β-L,L-ZAPM and α-L,L-ZAPM, as well as a blank polymer in which no print molecule was included, were prepared and evaluated in chromatographic mode.

The nonspecific binding of α-L,L-ZAPM and β-L,L-ZAPM, exhibited by the blank polymer B-1 shows that poly(MAA−4VPy−EDMA) itself, with the typical composition, retained the β-isomer longer than the α-isomer (Table 3), this becomes reasonable, considering that the latter has a lower acidity. After a template, α-L,L-ZAPM, is introduced for imprinting, the retention time of the α-isomer is increased more than that of the β-isomer, so that it gives no separation when a mixture of the two isomers is applied into columns packed with these α-isomer-imprinted polymers.

Table 2. Diastereo- and Enantiospecificities of Anti-β-L,L-ZAPM Bulk MIPs[a]

	k'			
polymer	α-L,L	α-D,D	β-L,D	β-D,L
β-1	1.2	1.2	2.6	2.3
β-2	1.2	1.2	2.7	2.0
β-3	0.9	0.9	1.4	1.3

[a] Polymer particles were packed into a 100 × 4.6 mm HPLC column. Acetonitrile containing 1% acetic acid was used as the mobile phase; acetone was used as the void marker. Capacity factor k' was determined by separately injecting 8.5 μg of analyte.

Table 3. Enhancement of Binding Specificities of Molecularly Imprinted Adsorbents[a]

		k'			
polymer	print molecule	α-L,L	β-L,L	α	f/g
α-1	α-L,L	2.2	2.0	1	0
B-1		0.4	0.7	1.5	0.1
α-2	α-L,L	1.5	1.6	1	0
α-3	α-L,L	0.8	0.9	1	0
β-1	β-L,L	1.2	3.0	2.7	1
β-2	β-L,L	1.2	4.1	3.4	1
β-3	β-L,L	0.9	1.6	1.6	0.9

[a] Polymer particles were packed into a 100 × 4.6 mm HPLC column. Acetonitrile containing 1% acetic acid was used as the mobile phase; acetone was used as the void marker. Capacity factor k' was determined by separately injecting 8.5 μg of analyte. Separation factors, α,and f/g values were calculated from injecting 8.5 μg of mixture composed of equal amounts of α-L,L and β-L,L.

(17) Mathews, C. K.; van Holde, K. E. *Biochemistry*, 1st ed.; Benjamin/Cummings: Redwood City, CA, 1990; Chapter 5.
(18) Sellergren, B. *Anal. Chem.* **1994**. *66*, 1578−1582.
(19) Martin, P.; Wilson, I. D.; Morgan, D. E.; Jones, G. R.; Jones, K. *Anal. Commun.* **1997**, *34*, 45−47.
(20) Matsui, J.; Okata, M.; Tsuruoka, M.; Takeuchi, T. *Anal. Commun.* **1997**, *34*, 85− 87.

Polymers β-1, β-2, and β-3 were prepared using β-L,L-ZAPM as a print molecule, and the separation factors of α-/β-L,L-ZAPM by using the obtained polymers as stationary phases were greatly increased. With the same polymerization recipe except for the print molecule, polymer β-1 produced α and f/g values of 2.7 and 1, respectively, compared with the values of 1.5 and 0.1 obtained from the blank polymer, B-1. Nonspecific binding can also be estimated by comparing the capacity factors of α-D,D-ZAPM and β-L,L-ZAPM, on polymers α-1 and β-1. Because neither of the analytes is the print molecule for polymer α-1, retention behavior can be supposed to result completely from the nonspecific effect. In contrast, the corresponding results on polymer β-1 will include imprinting contribution. The resulting capacity factors of α-D,D-ZAPM and β-L,L-ZAPM were 1.2 and 2.0, respectively, on polymer α-1, which changed to 1.2 and 3.0 when β-1 is substituted. A mixture composed of 8.5 μg of α-L,L-ZAPM, 8.5 μg of β-L,L-ZAPM, and 8.5 μg of β-D,D-ZAPM was also applied into a column packed with polymer β-2, and the chromatogram is shown in Figure 2a. Separation of β-L,L-ZAPM from α-L,L-ZAPM is much better than that of β-D,D-ZAPM from α-L,L-ZAPM, which from another aspect confirms our arguments. It is obvious that the binding specificities of the polymeric adsorbent have been increased by molecular imprinting. With the molar ratio of 4VPy/MAA of 1:1, the imprinted polymers gave baseline separation of the target synthetic molecule from the byproduct (Figure 2b).

Preparation and Characterization of Molecularly Imprinted Polymer Beads. The spherical MIPs have drawn much attention recently, as the regularly shaped particle morphology can confer better mass transfer in various applications. Another reason is that it is more practicable to use suspension polymerization for scale-up, e.g., for the mass production of imprinted adsorbents. The former is especially important when imprinted polymers are used as stationary phases in chromatography. Molecularly imprinted polymer beads have been successfully prepared by suspension polymerization, using imprinting recipes similar to that for the bulk MIPs.[10,11] Using a perfluorocarbon fluid as a dispersing phase and the same print molecule−functional monomer composition as the bulk polymer β-1, we also prepared a beaded polymer against β-L,L-ZAPM (Figure 3). When the obtained polymer beads were characterized in chromatographic mode, using acetonitrile containing 2% acetic acid as a mobile phase, they displayed the same binding characteristics as the imprinted bulk polymer and gave similar separations of the respective isomers. The result indicated that effective imprinting could be transferred onto polymer beads, using recipes optimized from the imprinted bulk polymers. It can be envisaged that these beaded MIPs could be more practicable in chromatographic applications, for either purification or analytical purposes.

Application of MIPs for Byproduct Removal in the Chemical Synthesis of Aspartame. Except for the rapid development of enzymatic techniques,[21] one of the most feasible chemical syntheses of aspartame on an industrial scale is the ring-opening reaction of N-protected L-aspartic anhydride with L-phenylalanine methyl ester depicted in Figure 1.[14] While enzymatic condensation of N-protected L-aspartic acid with L-phenylalanine methyl ester forms only the α-isomer, two kinds of isomers (α- and β-form) are produced by the chemical method. The byproduct, e.g. β-dipeptide, has to be removed from the objective product. It has been shown previously in chromatographic mode, that the anti-β-L,L-ZAPM polymers can specifically adsorb the print molecule. Extraction of this synthetic byproduct was further investigated. In a preliminary experiment, a dilute solution composed of the two isomers in acetonitrile with the molar ratio of α/β = 2.3 (which was proposed as a typical synthetic result in acetonitrile) was extracted with all imprinted polymers and a blank polymer, and the extraction effects are listed in Table 1. With the existence of nonspecific binding, all polymers adsorb more β-isomer than the other. Compared with blank polymer B-1, the two anti-β-L,L-ZAPM polymers, β-1 and β-2, extract more byproduct from sample solution than the two anti-α-L,L-ZAPM polymers, α-1 and α-2. Polymers α-3 and β-3 have selectivities inferior to that of other imprinted polymers. From the chromatographic analysis (Table 3), polymer β-2 should have higher extracting power than

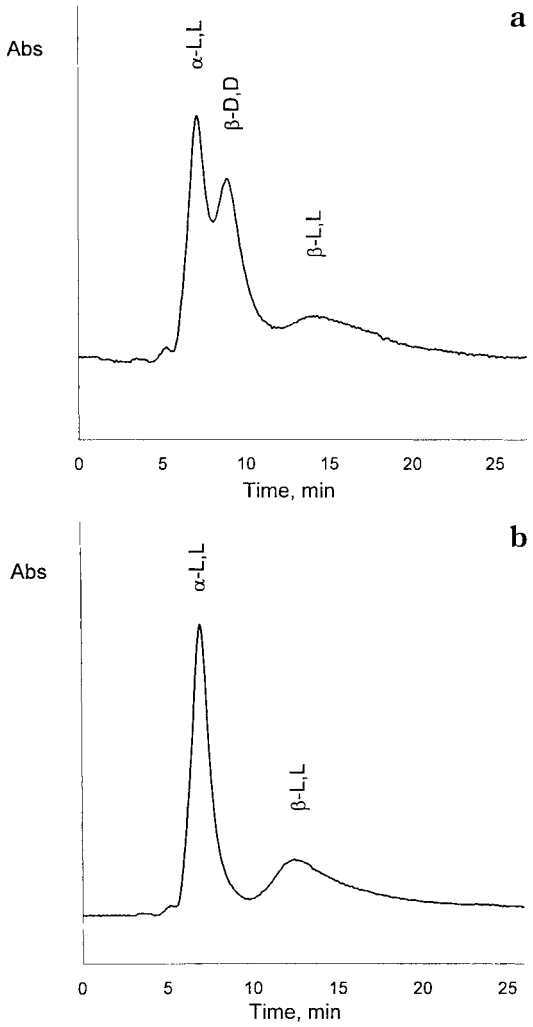

Figure 2. Separation of different isomers using an anti-β-L,L-ZAPM MIP (polymer β-2). (a) Separation of a 25.5-μg mixture composed of equal amounts of α-L,L-ZAPM, β-D,D-ZAPM, and β-L,L-ZAPM on a 100 × 4.6 mm HPLC column packed with polymer β-2. Acetonitrile containing 1% acetic acid was used as mobile phase with a flow rate of 0.5 mL/min. b) Separation of a 34-μg mixture composed of equal amounts of α-L,L-ZAPM and β-L,L-ZAPM on a 100 × 4.6 mm HPLC column packed with polymer β-2. Acetonitrile containing 1% acetic acid was used as mobile phase with flow rate of 0.5 mL/min.

(21) Nakanishi, K.; Kamikubo, T.; MAtsuno, R. *Bio/Technology* **1985**, *3*, 459−464.

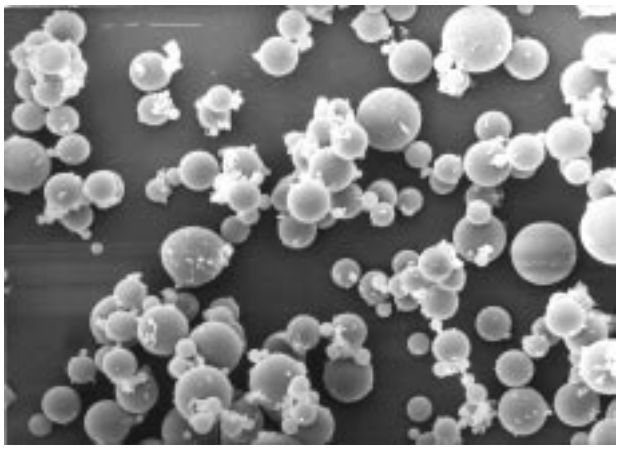

Figure 3. Scanning electron micrograph of the molecularly imprinted polymer beads. The image was obtained using an ISI 100A SEM at 25 kV with a magnification of 1000× (reproduced at 60% of original).

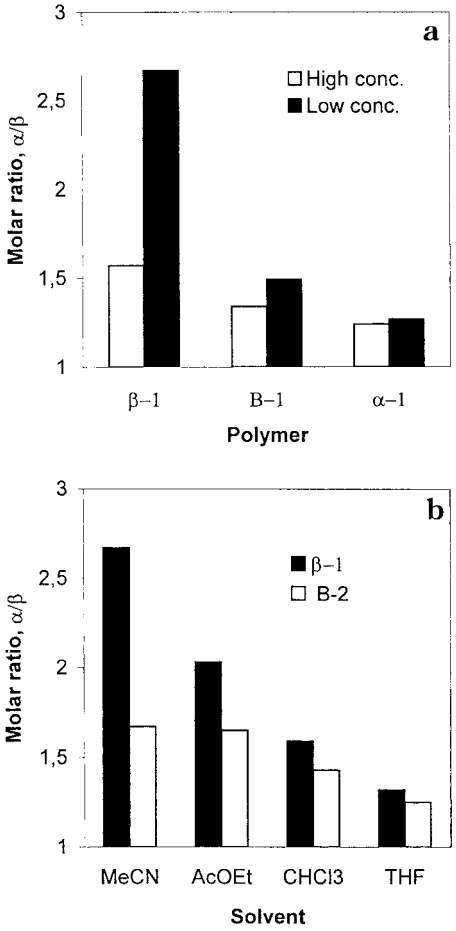

Figure 4. Influence of sample concentration and solvent on the specific extraction of print molecule by imprinted polymers. (a) For high sample concentration, 5 μmol of α-L,L-ZAPM and 5 μmol of β-L,L-ZAPM were incubated with 150 mg of polymer in 1 mL of acetonitrile for 1 h at room temperature. For low sample concentration, 0.5 μmol of α-L,L-ZAPM and 0.5 μmol of β-L,L-ZAPM were processed in the same way. (b) A mixture composed of equal amounts (0.5 μmol) of α-L,L-ZAPM and β-L,L-ZAPM was incubated with 150 mg of polymer in 1 mL of solvent at room temperature for 1 h. After centrifugation, the supernatant was analyzed by reversed-phase HPLC and the molar ratio of the components were calculated. Data are mean values of duplicate determinations.

β-1; however, this is not found in batch-mode extraction. The fact may be rationalized by the difference between the two experimental conditions. The mobile phase in chromatographic analysis has been modified by adding a small amount of acetic acid, which may play important roles in the adsorption/desorption process. On the other hand, the kinetic effect becomes less important when extractions are performed in batch mode.

Figure 4a shows the influence of the sample concentration on the extraction effects. The sample solution was composed of equal amounts of both the α-and β-forms. The difference between polymers β-1 and B-1, as well as the difference between α-1 and B-1, is greatly enlarged by the diluted sample solution. It is verified that with less ligand present, specific binding plays an important role in the solid-phase extraction. For practical usage, a compromise has to be made between binding specificity and loading capacity of imprinted adsorbents.

In addition to the blank polymer B-1, an anti-β-D,D-ZAPM polymer (polymer B-2) was also prepared and used as a reference. While polymer B-2 displayed higher adsorption capacities for both α- and β-isomers than did B-1, the nonspecific adsorption of β-isomer on the former was even higher compared with the α-form (data not shown). Thus polymer B-2 was also used as a reference in later experiments. The result of extraction performed in different solvents by using imprinted polymer β-1 and reference polymer B-2 is presented in Figure 4b. The specific adsorption of β-L,L-ZAPM by polymer β-1, as characterized by the difference between β-1 and B-2, is decreased from acetonitrile and ethyl acetate to chloroform and tetrahydrofuran, in the same order as a decreasing dielectric constant: acetonitrile > ethyl acetate > chloroform.[14] Solvents with low dielectric constant may be less preferable for the application of noncovalent imprinting employing ionic interactions.

For the investigation of the practical applicabilities of our MIPs for byproduct removal, the chemical synthesis of N-protected aspartame was performed in acetonitrile. The coarse product solution has the following molar composition: α-L,L-ZAPM, 59%; β-L,L-ZAPM, 19%; Z-L-aspartic acid, 22%. The diluted course solution was successively extracted with an anti-β-L,L-ZAPM polymer (β-1) and the polishing outputs are depicted in Figure 5. A reference polymer (B-2) was used in the same way to follow the nonspecific purification. After a 5-times extraction with polymer β-1, product purity was increased from 59 to 96%, while the reference polymer only provided a 87% final purity. The removal of the two contaminants by the anti-β-L,L-ZAPM polymer was demonstrated clearly to be more efficient than by the reference polymer. Z-L-Aspartic acid can be considered as a "hapten" for the anti-β-L,L-ZAPM antibody mimic, so the removal of this "hapten" is also facilitated by the partial complementary binding site from the artificial antibody. In the real case, adsorbents can be washed with the same solvent as used for extraction, and the collection can be repeatedly extracted. The imprinted polymer can also be regenerated by solvent extraction to remove adsorbed material for later usage. In some cases, the scavenged substance can be used for other purposes, for example, as new template for the production of MIPs as used in the present study. Finally, the stepwise batch operation can be substituted by chromatography, for example, using the beaded MIPs as stationary phases, which could greatly simplify all the procedures

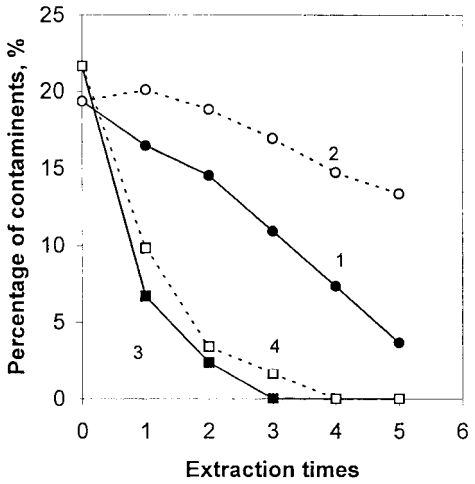

Figure 5. Purification of synthesized coarse product. The 10-times diluted reaction solution was repeatedly extracted with polymers β-1 and B-2 (150 mg of polymer/mL). After each step, the supernatant was analyzed by reverse-phase HPLC. Removal of contaminants was evaluated by the calculated molar composition. Data are mean values of duplicate determinations. Curves: (1) β-L,L-ZAPM, polymer β-1; (2) β-L,L-ZAPM, polymer B-2; (3) Z-L-Asp, polymer β-1; (4) Z-L-Asp, polymer B-2.

of sample application, elution, regeneration, and column reequilibration.

CONCLUSION

A noncovalent molecular imprinting technique has been applied for the preparation of diastereo- and enantioselective adsorbents for a dipeptide derivative. The binding specificities of the obtained polymers were enhanced by the introduction of complementary recognition sites by molecular imprinting. The present study verifies that, in addition to nonspecific binding, imprinted cavities, as well as the template-directed positioning of polymeric functionalities can be utilized to produce highly specific adsorbents for target substrate. Both methacrylic acid and 4-vinylpyridine were used as functional monomers in the imprinting against an acidic template. While the basic monomer can ionically interact with the free carboxylic group, the acidic monomer provides hydrogen bond interactions with additional "epitope" in the print molecule. This concerted complexation between the print molecule and the functional monomers resulted in an improved imprint memory for the obtained polymers.

The polymers imprinted against β-L,L-ZAPM displayed high specificity for the print molecule and can be used as downstream product polishers in the chemical synthesis of the synthetic precursor for an artificial sweetener, aspartame. The coarse product, containing both β-L,L-ZAPM and Z-L-aspartic acid was purified by solid-phase extraction of these two contaminants. Product purity was increased from 59 to 96% using repeated extractions. The maximum improvement of product purity from a single extraction step reached 20%. In the present investigation, because the byproduct, which is of the least commercial significance, is used as a print molecule, careful recovery of this template material becomes less important. The durable, regenerated MIPs can be used repeatedly for later applications. The molecularly imprinted polymer beads prepared using an optimized recipe retained the same binding characteristics as the bulk MIP, but also improved mass transfer for various separation processes. Such beaded MIPs can be envisaged for use in both production and analytical chromatography.

ACKNOWLEDGMENT

The authors thank Dr. Peter Cormack for linguistic advice.

Received for review January 26, 1998. Accepted April 13, 1998.

AC980069D