Combined Hydrophobic and Electrostatic Interaction-Based Recognition in Molecularly Imprinted Polymers

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ABSTRACT: Molecularly imprinted polymers selective for D- and L-phenylalanine have been prepared in water solution using 2-acryloylamido-2,2′-dimethylpropane sulfonic acid and bisacryloyl β-cyclodextrin as functional monomers, and *N*,*N*′-diacryloylpiperazine as cross-linker. Polymer−ligand recognition was evaluated using a combination of HPLC, displacement chromatography and fluorescence spectroscopic studies. HPLC studies revealed that the imprinted polymers possess optimal ligand selectivity in 30% water solution in acetonitrile. The results demonstrate that a balance of hydrophobic and electrostatic interactions is required for ligand recognition by these polymers. The imprinting effect is sufficiently strong to reverse the inherent chiral selectivity of the cyclodextrin molecule.

Introduction

The past 25 years have seen considerable effort being devoted to the development of synthetic systems capable of mimicing the highly specific molecular recognition and complementarity observed in Nature.[1] Molecular imprinting has evolved as a versatile technique for the preparation of recognition sites in synthetic polymers.[2] The utility of the technique, and the robustness of the highly cross-linked polymer products, has seen molecularly imprinted polymers (MIPs) applied in a range of application areas,[3] e.g., synthetic receptors/antibodies, synthetic enzymes and chromatographic stationary phases. The technique entails the use of functionalized monomers capable of forming reversible interactions with a template structure, the nature of these interactions determining the quality and quantity of recognition sites in the resultant polymer.[4] Polymerization in the presence of an inert cross-linking monomer traps the template−monomer complexes in a rigid polymer matrix. Extraction of the template under suitable conditions yields sites selective for the template structure.

To this point in time the general approach has been limited to template structures soluble in organic solvents. The capacity to utilize molecular imprinting for the preparation of synthetic receptors for water soluble (organic solvent insoluble) substances would be of great potential for the use of imprinted polymers in a range of biomedical and environmental diagnostic applications. The hydrophobic moiety selective recognition characteristics of the α-1,4 linked heptamer of glucopyranose, β-cyclodextrin (CD, Figure 1),[5] were envisaged as offering a means for surmounting this problem. Cyclodextrins have previously been shown to be capable of binding aromatic amino acids.[6] Here we report the first general approach for molecular imprinting in aqueous solution, where the hydrophobic selective binding capacity of CD-based functional monomers is employed in concert with electrostatic interacting functional monomers, as often is observed in biological recognition systems.[7] Polymers selective for the enantiomers of phenylalanine and reference polymers have been prepared and their binding and selectivity for the enantiomers of phenylalanine evaluated chromatographically and with fluorescence binding studies.[8]

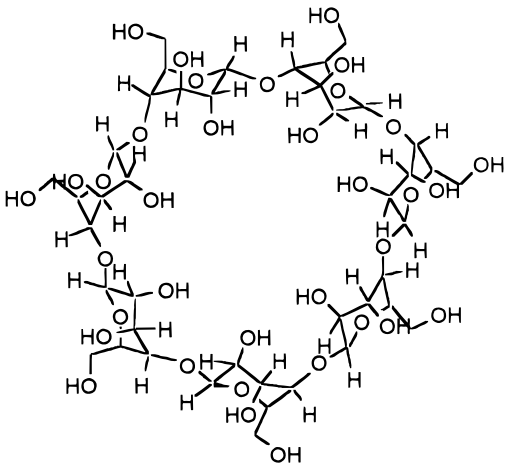

Figure 1. Structure of β-cyclodextrin.

Results and Discussion

The templates selected for this study were the enantiomers of phenylalanine; the zwitterionic nature of this amino acid precludes its use in traditional molecular imprinting protocols. The novel CD−monomer was prepared by the acylation of β-cyclodextrin with acryloyl chloride, ^{1}H NMR evaluation revealed a mixture of substitutional isomers with 1.9 acryloyl moieties per cyclodextrin. Molecularly imprinted polymers were prepared in the presence of either L-phenylalanine (**P(0)**) or D-phenylalanine (**P(1)**) by the $(NH_4)_2S_2O_8$/*N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) initiated polymerization of the functional monomers 2-acryloylamido-2,2′-dimethylpropane sulfonic acid (AMPSA) and bisacryloyl β-cyclodextrin (CD) and an excess of the water soluble cross-linker *N*,*N*′-diacryloylpiperazine. Water was used as the solvent and porogen (Table 1). Template selective recognition was envisaged to arise from a combination of binding of the template phenyl moiety into the hydrophobic cavity of the cyclodextrin, ion

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Table 1. Polymer Compositions[a]

polymer	AMPSA[b]	CD[c]	DAP[d]	D-Phe	water
P(1)[e]	0.8	0.4	16	0.4	200
P(B)	0.8	0.4	16		200
P(2)	0.8		16	0.4	200
P(3)		0.4	16	0.4	200

[a] All quantities are in mmol. [b] 2-acryloylamido-2,2′-dimethylpropane sulfonic acid. [c] β-Cyclodextrin monomer. [d] *N*,*N*′-Diacryloylpiperazine. [e] L-Phenylalanine MIP, **P(0)**, was prepared identically to **P(1)**, though with the substitution of L-Phe for D-Phe.

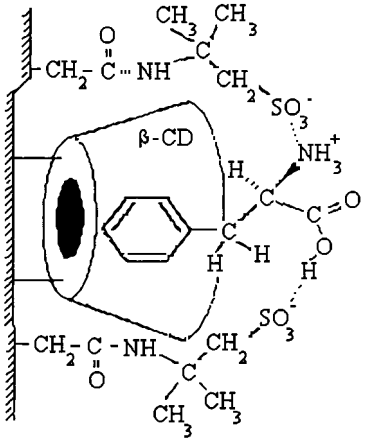

Figure 2. Schematic illustration of the binding of D-phenylalanine in an imprinted polymer composed of polymerizable β-cyclodextrin, 2-acryloylamido-2,2′-dimethylpropane sulfonic acid (AMPSA), and *N*,*N*′-diacryloylpiperazine.

pairing and hydrogen bonding of the amine and carboxyl to the sulfonic acid group of AMPSA, and van der Waals shape complementarity (Figure 2).

Initial efforts were directed toward studying the enantioselectivity of these polymers. The MIPs were evaluated with respect to their binding of the enantiomers of phenylalanine when employed as HPLC chiral stationary phases. In both cases the MIPs demonstrated selectivity for their respective templates, i.e. **P(0)** for L-Phe and **P(1)** for D-Phe, thus confirming that the molecular imprinting process functions for both these systems. Polymer imprinted with L-Phe (**P(0)**) yielded a separation factor of 1.15 when analyzed using optimized elution conditions (30% water in acetonitrile), whereas the D-Phe MIP, **P(1)**, produced a separation factor of 1.10 under the same conditions. The superior selectivity of **P(0)** over **P(1)** arises from the inherent chiral selectivity of the CD residues for the L-enantiomer. Thus, the observed selectivity of **P(1)** for D-Phe over L-Phe may be considered to arise purely from the contribution of the imprinting process. Most interestingly, the influence of the imprinting effect is sufficient to overcome the natural selectivity of the CD residues for the L-Phe. These observations were supported by frontal chromatographic analysis,[9] again using 30% water in acetonitrile, which revealed a K_{diss} for binding of D-Phe to **P(1)** of 1.5×10^{-3} M, with a site population (B_t) of 8 ± 2 mmol/g (dry weight) of polymer. The binding of L-Phe to **P(1)** was weaker by an order of magnitude and the site population was lower by 3 mmol/g (dry weight) of polymer, however more exact quantification of L-Phe binding was not possible due to the small differences in retention between the concentrations of L-Phe studied. As template recognition by **P(1)** is not augmented by inherent ligand selectivity, this system was selected for further study.

To establish more conclusively the nature of recognition in these systems, and the contribution of the functional monomers used, a series of imprinted and nonimprinted reference polymers was also prepared (Table 1). The ligand enantioselectivities of **P(1)** and the reference systems were again examined by employing the polymers as HPLC chiral stationary phases using a range of eluent mixtures, from 10 to 100% water in acetonitrile (Table 2). The capacity factors were highly reproducible, with an error of ± 0.01. As expected, polymer **P(B)**, identical to **P(1)** but prepared in the absence of template, showed no imprinting effect, only the inherent chiral selectivity of the β-cyclodextrin for L-phenylalanine. In the case of **P(3)**, which was imprinted with D-Phe using only the cyclodextrin-based monomer, i.e. devoid of the electrostatic interacting monomer AMPSA, the absence of complementary electrostatic interactions does not allow for sufficient selectivity to overweigh the inherent selectivity of the CD moiety for the L-form in favor of the template in this polymer.

P(2), prepared in the absence of the CD monomer and thus allowing for only electrostatic interactions between the template and AMPSA, did induce an imprinting effect and demonstrated low specificity for D-Phe in solutions containing a high percentage of acetonitrile, the lower buffer polarity thus augmenting the hydrogen bonds. Other ionic interactions are the major driving forces in the complex formation between polymer binding sites and phenylalanine in nonpolar media. In aqueous media, however, recognition effects based on these interactions are significantly weakened.[10] This polymer and the observed results reflect the influence of purely electrostatic interaction based molecular imprinting. Accordingly, polymer **P(1)**, containing both functional monomers, CD and AMPSA, and imprinted with D-Phe, demonstrated superior selectivity to the reference polymers, with optimal selectivity for the template being observed in 30% water. It may therefore be concluded that a balance of hydrophobic and electrostatic interactions is essential for selectivity for D-Phe by **P(1)**. A significant difference exists in the mechanisms underlying the ligand selectivities of **P(1)** and **P(3)** as evidenced by the respective buffer−selectivity profiles.

In addition to the investigation of chiral recognition properties, the selectivity of the MIPs for a range of structures related to phenylalanine was also investigated. In 30% water in acetonitrile, both **P(1)** and the nonimprinted polymer **P(B)** show different selectivities for Boc−D-Phe, D-Phe, and D-Phe−NH_2 (Figure 3). Notably, a marked reversal of the rank order of elution of Boc−D-Phe and D-Phe was observed. On the nonimprinted polymer **P(B)**, the randomly distributed CD residues provide binding opportunities for the hydrophobic *tertiary*-butyl group of Boc−D-Phe, whereas in **P(1)** the steric bulk of Boc−D-Phe does not permit access to the CD residues which are involved in the more selective, higher fidelity, D-Phe recognition sites.

Displacement chromatographic studies were performed to provide an estimate of the degree of enantioselectivity of the imprinted polymer **P(1)**.[11] The method relies upon the competitive displacement of an organic dye from polymer binding sites. In this case we used 4-nitrophenol as a marker because of its strong affinity for the cyclodextrin. After incubation of the polymer with eluent containing 4-nitrophenol, samples of D- or

Table 2. Recognition of D- and L-Phe by Polymer HPLC Stationary Phases

	P(1)			P(B)			P(2)			P(3)		
water, %	κ'_D	κ'_L	α	κ'_D	κ'_L	α	κ'_D	κ'_L	α	κ'_D	κ'_L	α
10[a]	9.24	9.10	1.02	6.76	7.00	0.97	17.5	16.0	1.09	32.0	33.4	0.96
20	1.59	1.54	1.03	1.48	1.50	0.99	1.63	1.58	1.03	3.17	3.26	0.97
30	1.07	0.97	1.10	0.98	1.00	0.98	1.02	1.02	1.00	1.10	1.12	0.98
50	0.22	0.21	1.05	0.40	0.40	1.00	0.49	0.49	1.00	0.39	0.42	0.93
70	0.07	0.08	0.88	0.10	0.10	1.00	0.07	0.07	1.00	0.28	0.29	0.97
100	0.05	0.06	0.83	0.11	0.11	1.00	NR[b]	NR[b]		NR[b]	NR[b]	

[a] On account of the solubility of phenylalanine, it was not possible to evaluate these polymers with 100% organic phase. [b] Analyte was not retained on either column under these elution conditions.

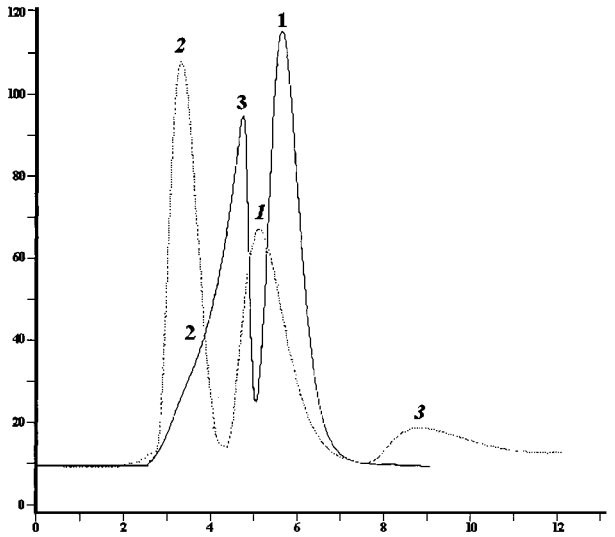

Figure 3. Chromatographic responses for D-phenylalanine imprinted (**P(1)**, dark line) and nonimprinted (**P(B)**, gray line) ligand selectivities: (**1**) D-phenylalanine; (**2**) *tert*-butyloxycarbonyl-D-phenylalanine; (**3**) D-phenylalanine amide. Eluent: H_2O/acetonitrile (30:70, v/v).

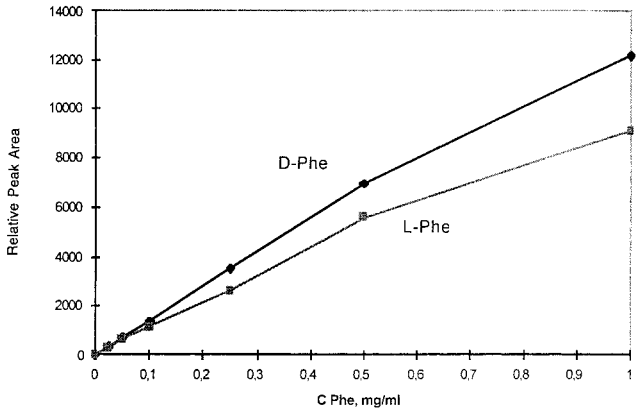

Figure 4. Dependence of the peak area of the 4-nitrophenol displaced by phenylalanine as a function of amino acid concentration.

L-Phe were injected onto the column, leading to competition for polymer binding between the analyte and 4-nitrophenol. The influence of phenylalanine sample concentration on the area of the peak from the displaced nitrophenol is illustrated in Figure 4. The affinity of the polymer binding sites is greater for D-Phe than for L-Phe. Interestingly, this difference increases with increasing amino acid analyte concentration, which may suggest the presence of sites selective for template complexes due to dimers and higher order complexes of the template, as has been indicated in recent studies of other molecularly imprinted polymer systems.[12]

The fluorescence of the dansyl group is known to be very sensitive to changes in media polarity and has been previously used for the investigation of polymer structure.[13] Polymer suspensions were equilibrated with aqueous solutions of dansylglycine and the shift in fluorescence emission, resulting from ligand−polymer binding was monitored. Clear differences were observed between the emission spectra of all polymers studied, indicating the presence of differences in the polarities of the ligand binding sites. **P(3)**, containing only the cyclodextrin functional monomer, produced the largest shift in fluorescence emission, from 550 nm (dansylglycine in water) to 530 nm. **P(2)**, with no hydrophobic cavity containing cyclodextrin monomer, produced a slight shift, from 550 to 552 nm, characteristic of a polar ligand binding environment. The nonimprinted polymer **P(B)** induced an intermediate fluorescence emission peak shift, from 550 to 540 nm, as could be expected from the presence of both electrostatic and hydrophobic interacting functional monomers, albeit randomly displaced throughout the polymer. The size of the shift is indicative of hydrophobic interactions providing a major contribution to binding. The imprinted polymer **P(1)**, however, with an monomer composition identical with that of **P(B)**, demonstrated a smaller apparent hydrophobic interaction based contribution to binding, which suggests that the mode of ligand binding is dependent upon both hydrophobic and polar interactions. This indicates that sites containing both the CD and AMPSA functional groups, as shown in the highly schematic representation of a polymer recognition site in Figure 2, are present in **P(1)**.

Conclusion

In this study, we have presented the first rational attempt to use the hydrophobic effect for preparing molecularly imprinted polymers and have shown its use in conjunction with electrostatic interactions for molecular imprinting in water. Chromatographic and fluorescence based recognition analyses have demonstrated the reliance upon both these types of interaction for optimal ligand binding. This new approach significantly broadens the scope of molecular imprinting through allowing for the development of specific sorbents for templates insoluble in organic solvents and opens the way to new possible application areas, especially for producing alternatives to biological receptor systems.

Experimental Section

General Information. Chemicals and solvents were of analytical or HPLC grade.

Synthesis of Bisacryloyl *β*-Cyclodextrin (1). To a stirred 0 °C solution of *β*-cyclodextrin (1 equiv) dissolved in freshly distilled pyridine was added dropwise an ice chilled solution of acryloyl chloride (2.2 equiv) in diethyl ether. The solution was slowly warmed to room temperature then stirred for 16

h. Evaporation of solvent under reduced pressure afforded a white solid, which after recrystallization from ethanol gave the product in 75% yield (mp 198−205 °C dec). ^{1}H NMR analysis revealed the product to consist of a mixture of substitutional isomers, with 1.9 acryloyl substitutions per β-cyclodextrin, and the IR spectrum of **1** showed a characteristic α,β-unsaturated carbonyl absorbance at 1720 cm^{-1}.

Polymer Syntheses. A series of molecularly imprinted and reference polymers was prepared, Table 1. In a typical imprinted polymer preparation, the template, D- or L-phenylalanine, was dissolved in water in a 50 mL screwcap borosilicate glass vial. The appropriate amounts of monomer(s) and cross-linker were added, and the tube was purged thoroughly with nitrogen before addition of the initator system: $(NH_4)_2S_2O_8$ (200 μL, 50%) and TEMED (50 μL). After sonication (5 s) of the reaction mixture, polymerization was carried out at 75 °C for 18 h. The resultant polymer was crushed and ground and particles were collected by sedimentation and filtration through sieves (63 μm and 25 μm) with water. The polymers were washed with water (100 mL ×10) to remove unreacted monomers and residual template. Finally, the polymer suspension (25−63 μm) was used for packing of HPLC columns. Polymer particles ≤25 μm were used for fluorescence measurements. The reference polymers, **P(0)**, **P(2)**, and **P(3)**, were similarly prepared and processed, though in the case of **P(B)** in the absence of template.

Polymer Physical Characterization. Combustion analyses were performed by Mikrokemi AB (Uppsala, Sweden). Surface area analysis was performed on a Micrometrics Flowsorb II 2300 instrument, using 30% N_2 in Ar. **P(1)**: C, 56.5; H, 7.5; N, 12.8; S, 0.7. BET surface area, 362 m^2/g; micropore area, 42.4 m^2/g, micropore volume, 0.014 cm^3/g; average pore diameter, 80.2 Å. **P(B)**: C, 56.1; H, 7.5; N, 12.8; S, 0.7. BET surface area, 324 m^2/g; micropore area, 37.3 m^2/g, micropore volume, 0.013 cm^3/g; average pore diameter, 80.0 Å. **P(2)**: C, 54.2; H, 7.7; N, 12.4; S, 0.7. BET surface area, 454 m^2/g; micropore area, 111.5 m^2/g, micropore volume, 0.046 cm^3/g; average pore diameter, 80.3 Å. **P(3)**: C, 58.4; H, 7.6; N, 13.4. BET surface area, 321 m^2/g; micropore area, 42.9 m^2/g, micropore volume, 0.015 cm^3/g; average pore diameter, 64.9 Å.

High Performance Liquid Chromatography. Polymer particles were packed into stainless steel HPLC columns (100 × 4.6 mm i.d.), using an air-driven fluid pump (Haskel Engineering Supply Co.). Columns were packed at 340 bar using water. Polymers were washed on-line with water until a stable baseline was obtained. Chromatographic experiments were performed using a Perkin-Elmer HPLC system. Samples containing D-Phe or L-Phe (1 mg/mL) were prepared in the eluent and 50 μL of solution was injected for analysis. Analyses were run at a flow rate of 0.4 mL/min with detection at 257 nm, using mobile phases consisting of water mixed with various amounts of acetonitrile. All reported chromatographic data represent the results of at least 4 concordant experiments. Capacity factors (κ') were determined from $\kappa' = (V - V_o)/V_o$, where V is the retention volume of a given species and V_o is the retention volume of the void marker (acetone). Effective enantioseparation factors (α) were calculated from the relationship $\alpha = \kappa'_D / \kappa'_L$, where κ'_D and κ'_L are the capacity factors of the D- and L-Phe, respectively.

Frontal Chromatography. Frontal chromatographic analysis[9,14] was performed on **P(1)** as follows: duplicate 5 mL injections of 1.5, 3, 5, 12, and 25 mM solutions were injected at a flow rate of 0.4 mL/min at room temperature (21−23 °C). Acetone was employed as the void marker, and the ultraviolet absorbance was monitored in the wavelength interval 257−270 nm.

Displacement Chromatography. An HPLC column packed with **P(1)** was equilibrated in a mobile phase containing 4-nitrophenol (10 μg/mL) and triethylamine (0.01%, v/v) in 20% water in acetonitrile until a steady baseline was achieved. Samples containing various concentrations of D- and L-Phe were prepared in eluent solution and volumes (50 μL) were injected for analysis. The signal was monitored at 410 nm and the area of the resulting peak, resulting from 4-nitrophenol displacement by phenylalanine, was recorded.

Fluorescence Measurements. A typical fluorescence experiment is described below: polymer (2 mg) was suspended in a solution of dansylglycine in water (3 mL, 33 μM). After incubation for 20 min at room temperature, the fluorescence of the suspension was measured (Hitachi F-2000 fluorescence spectrofluorometer). The excitation wavelength used was 355 nm and the fluorescence was detected at 420−560 nm.

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