Investigations into the Mechanisms of Molecular Recognition with Imprinted Polymers

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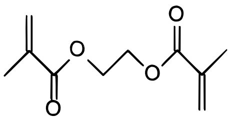
Received September 11, 1998; Revised Manuscript Received April 6, 1999

ABSTRACT: The nature of molecular recognition in an imprinted polymer that is formed by the self-assembly of binding monomer and imprint through noncovalent interactions is investigated. The system studied uses ethylene glycol dimethacrylate, methacrylic acid, and L-phenylalanine anilide as the cross-linking monomer, binding monomer, and imprint, respectively, to assemble the imprinted polymer. A proposal for the self-assembly mechanism between the binding monomer and imprint that occurs during polymer synthesis is derived from a single-crystal X-ray structure of a crystal containing binding monomer and imprint and from ^{1}H NMR and FTIR spectroscopy of solutions of these components. These studies show the presence of a salt-bridge interaction that leads to a 1:1 molecular complex between methacrylic acid and L-phenylalanine anilide and provide no evidence for the formation of higher-order molecular complexes of these species. Furthermore, macroscopic phase separation is observed between the imprint and binding monomer and the cross-linking monomer in this system prior to and after polymerization. These findings suggest a mechanism for enantioselective molecular recognition in the imprinted polymer involving remaining, occluded imprint molecules, which can provide for binding via imprint−imprint intermolecular interactions. Support for this mechanism is provided from polymers prepared using a combination of imprint and mimic, L-phenylalanine 4-vinylanilide; the latter remains covalently bound in the polymer and is shown to increase the rebinding of imprint while not significantly affecting the binding of the opposite enantiomer. The proposed mechanism provides insight into the nature of binding site heterogeneity in imprinted polymer systems.

Introduction

A frequently used method for the molecular imprinting of materials is that of noncovalent self-assembly.[1] The major advantage of this approach is its ease of implementation due to the fact that the imprinted site self-assembles during the synthesis process to generate binding interactions that are reversible and in many ways similar to interactions used by natural systems such as enzymes and antibodies (Scheme 1).[2] However, despite the growing acceptance of the method of noncovalent, molecular imprinting by self-assembly as a strategy for preparing templated materials, a significant problem that remains inherent to these materials is a strong binding site heterogeneity.[3] The objective of our work is to study the origins of binding site heterogeneity in these imprinted polymers by understanding the molecular level phenomena governing the imprinting effect and the subsequent capability of the imprinted polymer to perform molecular recognition. To this end, we investigated one of the most studied noncovalent, molecular imprinting systems that was originally introduced by Mosbach and colleagues.[4] The system is comprised of a hydrophobic cross-linking monomer, ethylene glycol dimethacrylate **1**, that serves as the mechanical support for the imprinted site and a binding monomer, methacrylic acid **2**, that is proposed to form favorable hydrogen bonding and electrostatic interactions with the imprint L-phenylalanine anilide **3** (L-PheNHPh). Imprinted polymers that utilize the imprint L-PheNHPh have reported enantioselectivities of up to 13 (defined as the ratio of the chromatographic capacity factors of the L- and D-enantiomers),[5] and other polymers using **1** and **2** have been reported to function as imprinted catalysts[6] that have shown enantioselectivity.[7] The primary goal of our work is to determine whether noncovalent, molecular imprinting by self-assembly in the L-PheNHPh system fundamentally gives rise to isolated molecular binding sites (Scheme 1) or whether phase separation can occur to give a composite binding site comprising a lattice associated with one another via imprint−imprint intermolecular interactions (Scheme 2) or a combination of both situations. In contrast to the mechanism represented in Scheme 1, where several imprinted fragments of binding monomer interact with an *isolated* imprint molecule in a concerted fashion, molecular recognition in Scheme 2 is provided for by interactions between adjacent imprint molecules and a single-point binding interaction between imprint and binding monomer fragment. In Scheme 2, a fraction of imprint that remains in the material after extraction and is surrounded by open void space (containing binding monomer fragments positioned for single-point interaction) can serve as a center of nucleation for readsorption. It is important to note that materials capable of chiral molecular recognition of amino acid derivatives based on an association mechanism similar to that represented in Scheme 2 have been reported previously.[8] Thus, in ultimately distinguishing between the modes of molecular recognition in an imprinted material as they relate to Schemes 1 and 2, it is important to determine whether the imprint is homogeneously distributed throughout the material or whether it is clustered on some length scale that is accessible to a readsorbing species.

10.1021/ma981445z CCC: $18.00



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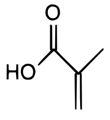

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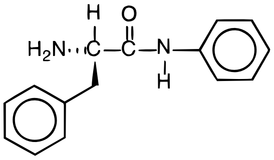

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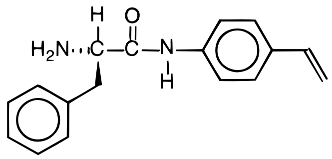

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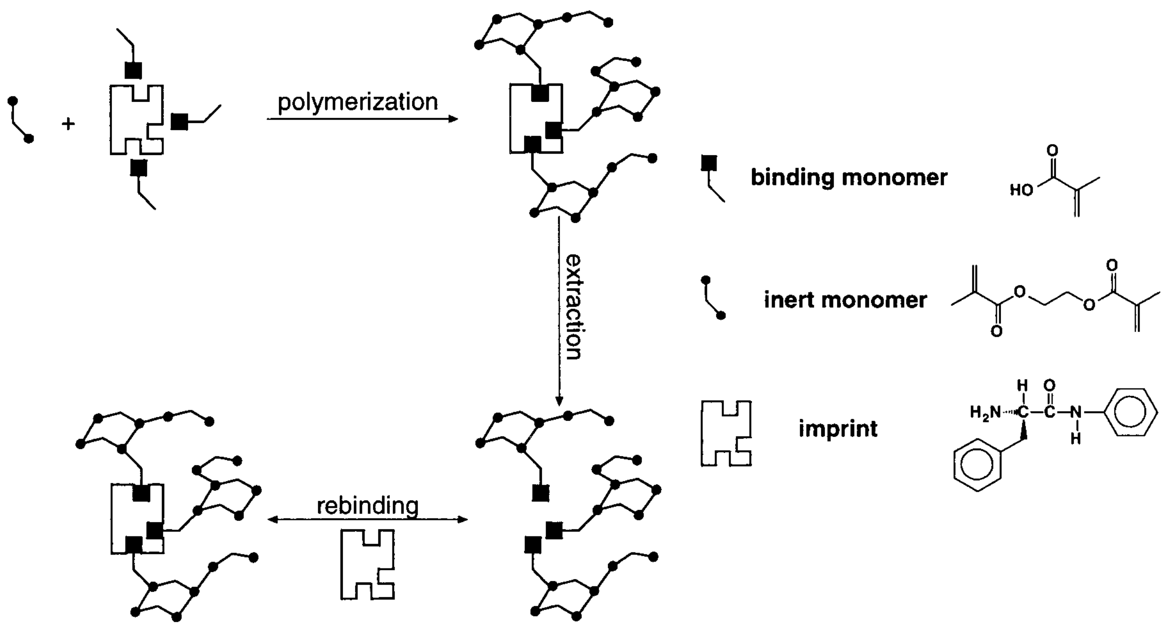
To address this issue experimentally, we prepared a vinyl-containing mimic of the imprint **3**, the 4-vinylanilide derivative of phenylalanine, **4**. This mimic was chosen because of its analogy to **3** in terms of possible functional group interactions with **2**, its size complementarity to **3** and because it can be covalently incorporated into the imprinted material upon polymerization. Thus, the addition of a small amount of **4** to the polymerization process allows some control over the amount of *isolated* imprint remaining in the polymer following imprint extraction. For the mechanism represented by Scheme 1, the addition of a small fraction of **4** to **3** as imprint into a molecularly imprinted polymer should slightly decrease the observed enantioselectivity and imprint adsorption capacity as compared with a polymer prepared only with **3**, since there are fewer open binding sites available due to the presence of the covalently bound and unextractable imprint **4**. For the mechanism represented by Scheme 2, the addition of **4** should cause a significant increase in the observed enantioselectivity and imprint adsorption capacity, since there are more nucleation centers available for molecular recognition. The increase implied in the enantioselectivity and adsorption capacity with the number of nucleation centers in Scheme 2 is a consequence of the continuous overloading of binding sites in this system under the conditions investigated, as discussed previously.[3d-g]

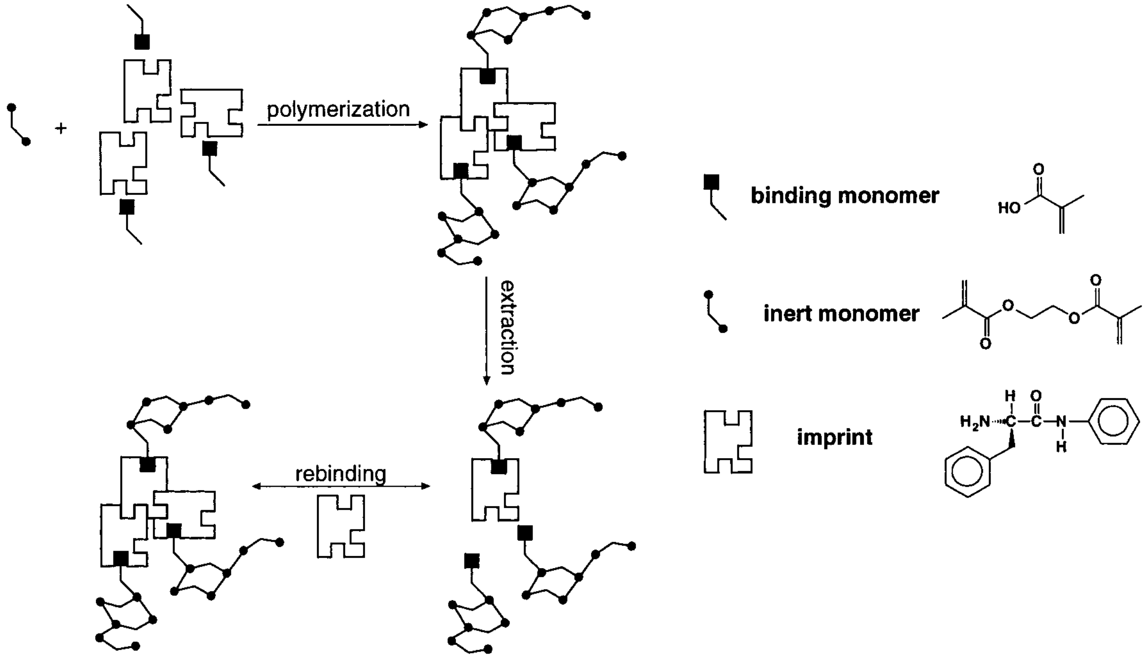
Results and Discussion

Characterization of the Self-Assembly Process. The interaction between L-PheNHPh and **2** has been investigated previously.[4] It was suggested that the binding monomer **2** and the imprint **3** self-assemble to form 3:1 complexes in the solution phase (in equilibrium with 2:1 and 1:1 complexes) prior to polymerization. These interactions were argued to be conserved during the polymerization process to subsequently provide the basis for molecular recognition in the final imprinted material according to the mechanism depicted in Scheme 1.[9] Here, we collected ^{1}H NMR spectra from titrating up to 5 equiv of **2** with **3** at four different temperatures in both deuterated acetonitrile and chloroform (at the concentration of **3** corresponding to an imprinted polymer synthesis prior to polymerization). The most significant change in the ^{1}H NMR chemical shift (NMR data included in Supporting Information) occurs from the exchangeable proton of the ion pair between **2** and **3** as observed previously.[4] The ^{1}H NMR titration experiments serve as a sensitive calibration for determining the number of acid equivalents in a solution of **2** and **3** (vide infra). FTIR can be used to analyze for the possibility of other hydrogen-bonding interactions besides the ion-pair interaction discussed above.[10] The carbonyl vibration of **3** was monitored by FTIR spectroscopy when titrating up to 5 mol equiv of **2** at room temperature in chloroform solution (FTIR data included

Scheme 1. Illustration of Molecular Imprinting by Noncovalent Self-Assembly Assuming That the Imprint Molecules Are Isolated from Each Other



Scheme 2. Illustration of Molecular Imprinting by Self-Assembly Assuming That the Imprint Molecules Are Not Isolated from Each Other; in This Scenario, in Addition to Having Interactions between Binding Monomer and Imprint, Intermolecular Interactions between Adjacent Imprint Molecules Are Also Significant



in Supporting Information). It is well established that hydrogen bonding has the general effect of broadening and lowering the frequency of the carbonyl stretch.[11] The results indicate that there is little effect of acid addition on the carbonyl band of **3**, as the frequency and width of the **3** carbonyl band remain virtually identical (within a tolerance of ±3 cm^{-1}) over the full concentration range of **2** investigated.[12] The spectroscopic data above provide no evidence for hydrogen-bonding interactions between **2** and **3** other than the single-point salt-bridge interaction. We note that it is not possible to rigorously dismiss the presence of other hydrogen-bonding interactions separate from the salt-bridge interaction solely on the basis of the spectroscopic evidence above, as the lack of spectroscopic signature upon acid titration may be a consequence of a transition in the mode of hydrogen bonding in this system from intramolecular to intermolecular.

Upon addition of all monomers and free-radical initiator, phase separation can be observed optically in this system. As can be seen in Figure 1 (a small amount of chlorophenol red dye was used to help visualize the distribution of imprint **3** in the imprinted polymer mixture prior to polymerization), the mixture prior to polymerization is separated into two phases: one rich in imprint **3** that is stained dark by the dye (bottom) and one containing little imprint **3** that is clear (top). Figure 2 shows the visual appearance of an as-made L-PheNHPh imprinted polymer. In our hands, the degree of visually observable phase separation in a L-PheNHPh imprinted polymer varied from none (clear) to almost complete phase separation as shown in Figure 2, and we have found that small variations in the experimental procedure (such as the extent of dryness of solvents and monomers used in the imprinted polymer syntheses) can cause variability in the results (precise details therefore provided in the Experimental Section). It is important to note in this regard that the materials used to collect adsorption data in all of the work presented here were from optically clear polymers (photographs provided in the Supporting Information).

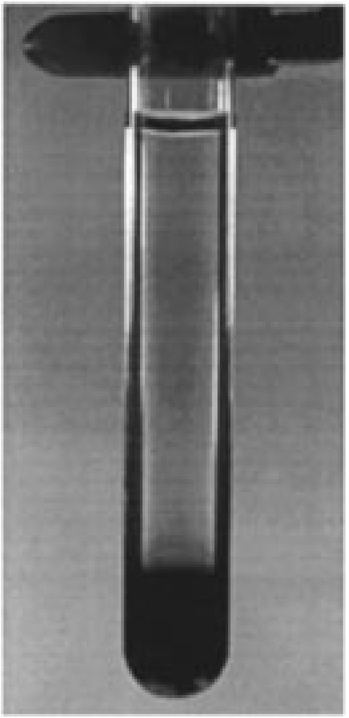


Figure 1. Optical photograph of a L-PheNHPh imprinted polymer synthesis mixture prior to polymerization. A small quantity of chlorophenol red dye was added to facilitate the visualization of the **2**−**3**-rich phase (lower layer) separate from the **1**-rich phase (upper layer).

It is possible to isolate the needles precipitated from within the polymers shown in Figure 2. (Some needles float to the top of the polymer during polymerization and can be subsequently scraped off with a spatula.) Liquid-phase ^1H NMR spectra of the needles dissolved in deuterated chloroform suggest that the needles are comprised of **2** and **3** in a molar ratio of 1:1.[13] The ^1H and ^{13}C NMR spectra from the solution obtained by dissolving the needles in deuterated chloroform provide

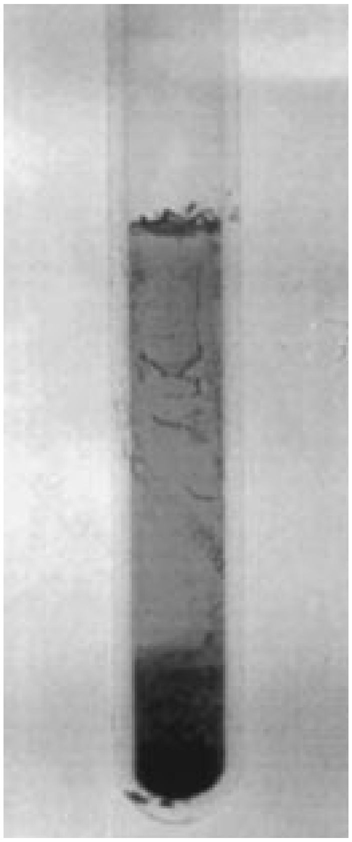


Figure 2. Optical photograph of an as-made L-PheNHPh imprinted polymer. The phase separation in this case is localized to upper and lower layers as in the case preceding polymerization represented in Figure 1.

no evidence for the incorporation of **1** into the collected needles and suggest that **2** and **3** can phase separate from **1** in the imprinted polymer, as observed in the liquid phase prior to polymerization. To further characterize the complex formed between **2** and **3**, single crystals were grown in a more controlled manner from a chloroform solution containing 4 mol equiv of **2** relative to the concentration of **3**.[14] Figure 3 shows an ORTEP plot and a stereoscopic representation for the structure solution of the crystal of **2** and **3**. (Crystallographic data are included in the Supporting Information.) The 1:1 stoichiometric composition of the crystal is consistent with the solution ^{1}H NMR and FTIR spectroscopic results discussed above. The 3 mol equiv of methacrylic acid remaining in the solution phase was confirmed by ^{1}H NMR experiments on the mother liquor after crystallization, indicating that acid in amounts greater than 1 equiv is excluded from the **2**−**3** crystal under the conditions investigated. It is important to emphasize for comparison that there are other examples of self-assembled crystal systems that are comprised of acid−base building units and where more than 1 equiv of acid is found to incorporate into the crystal via additional interactions.[15] To summarize information from the X-ray crystal structure solution, in the **2**−**3** crystal, there is a single-point binding between **2** and **3** via the salt bridge made by the acid proton and the amine, and there are no other independent interactions evident between **2** and **3**. In terms of transferring chiral information between molecules in the crystal, the bond distances in Figure 3 show that there are two intermolecular interactions that could potentially be responsible for this: a hydrogen-bonding interaction between O1 and the H of N2 (labeled H22N in Supporting Information) on an adjacent imprint molecule (fixes the position of the carbonyl oxygen) and an additional interaction between O1 and H of C15 (labeled H15 in Supporting Information) on an adjacent imprint molecule (fixes the position of the benzyl group).[16] These three intermolecular interactions (one between binding monomer and imprint and two between imprint and imprint) could be used to provide for chiral molecular recognition in a manner consistent with Scheme 2.

Polymer Prepared with Imprint Mimic. To obtain insight into the mechanism of molecular recognition exhibited by the L-PheNHPh imprinted polymer, the significance of remaining imprint in the polymer after extraction was investigated. By synthesizing imprinted polymers without (**P1**) and with (**P2**) the synthetic mimic of **3** that is covalently retained in the imprinted material following extraction, **4**, the influence of remaining imprint can be ascertained. Wulff et al. used **4** as a mimic since it is close in terms of size and chemical functionality to the imprint **3**.[17] The approach here differs from Wulff et al. in that the mimic **4** is used in combination with **3**. Below, the complementary roles of the imprint and mimic are discussed for the mechanism depicted in Scheme 2. The mimic **4** can dissolve within a cluster of **2** and **3** at the self-assembly stage of imprinting.[18] This is facilitated in the present study by using a relatively small amount of **4** as imprint (100 ppm) in conjunction with **3**, since at these small fractions of **4**, the phase separation of **4** from **3** that might occur at higher amounts is minimized. Upon polymerization, both **2** and **4** are covalently incorporated into the imprinted material. (The symbols **2′** and **4′** will be used to refer to the covalently incorporated molecules of **2** and **4**, respectively.) It is expected that **4′** participates in similar intermolecular bonding to **2′** and adjacent molecules of **3**, as does **3**.[18] Upon extraction, the imprint molecules of **3** surrounding **4′** are removed. This results in regions of isolated **4′**. These *isolated* molecules of **4′** that are covalently bound to the polymer framework and interact with **2′** function as centers for rebinding in the mechanism of Scheme 2. If, on the other hand, the molecular recognition mechanism resembles that of Scheme 1, some of the imprinted binding pockets will be lost due to the covalent binding of **4′**. However, the addition of 100 ppm of **4** should have a small effect on the observed adsorption capacity and enantioselectivity.

Three imprinted polymers were synthesized, and they are described in Table 1: **P1** is a reference polymer imprinted with **3**, **P2** is an imprinted polymer comprised of 100 ppm **4** and the remainder **3** as imprint, and **P3** is a reference polymer imprinted solely with **4**. Although previous investigations have shown little correlation between polymer morphology (pore size and pore volume) with imprinted polymer performance (selectivity and capacity) for the L-PheNHPh imprinted polymer system,[3g] we note that all polymers investigated here have the same morphology as ascertained by scanning electron microscopy and are macroporous as judged by nitrogen adsorption measurements. (**P1**, **P2**, and **P3** have BET surface areas within experimental error of each other and were on the order of 4 m^2/g.) It is noteworthy that the percentage of **4** extracted from **P3** is of the same order of magnitude as the percentage of unreacted double bonds after complete monomer conversion in similar cross-linked systems.[19] This result

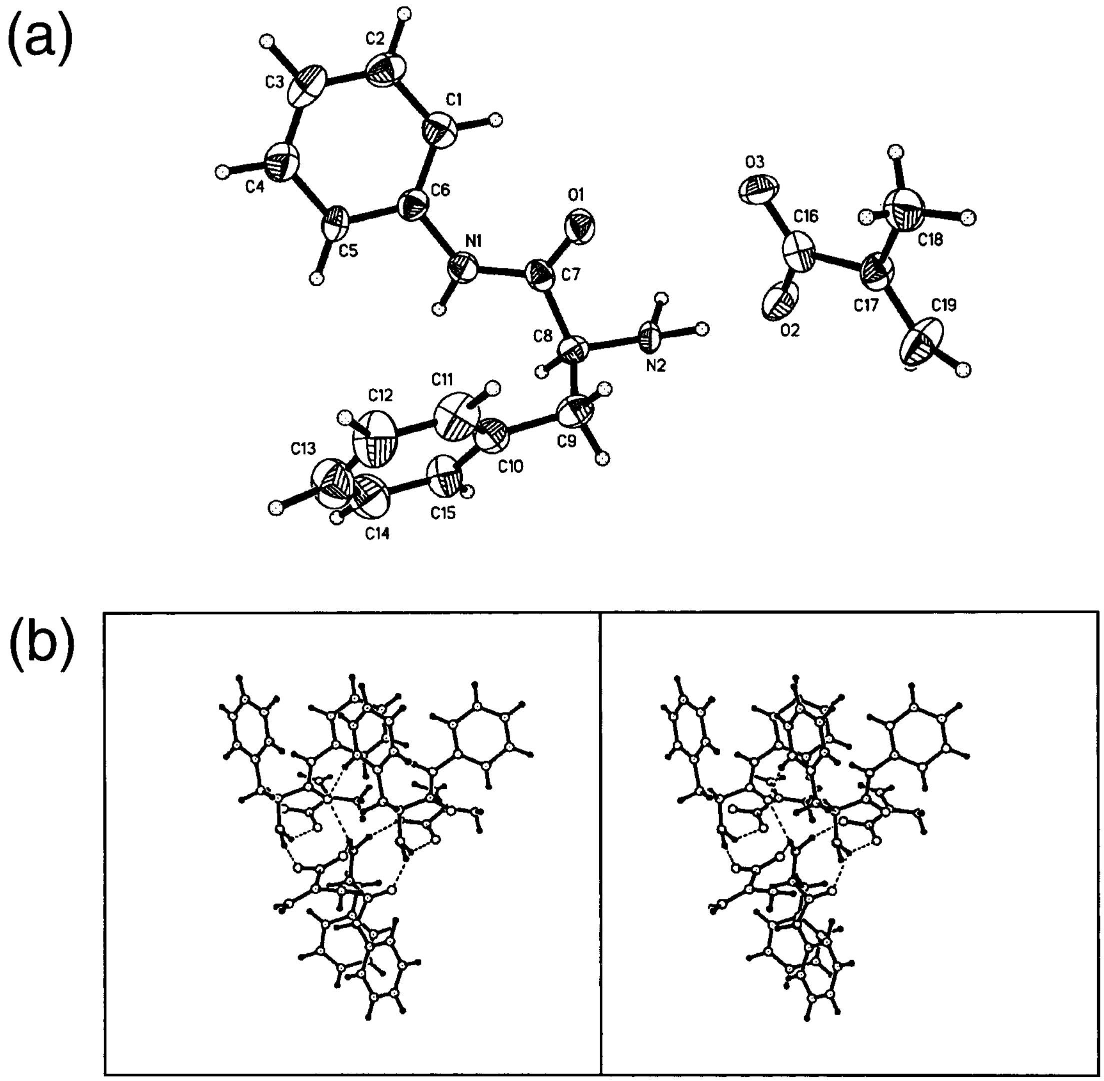


Figure 3. (a) ORTEP plot of the structure and atom labeling scheme for the **2**−**3** crystal. Thermal ellipsoids are drawn at the 30% probability level, and labels for hydrogen atoms are omitted for clarity. (b) Stereoscopic representation of the intermolecular bonding involved in the **2**−**3** crystal. Interatomic distances were used to identify hydrogen bonds, and these are labeled with dashed lines.

Table 1. Summary of Synthesized Imprinted Polymers

polymer	composition of imprint (mole)	% imprint extracted
P1	reference polymer, 100% **3**	93.2
P2	99.99% **3** and 0.01% **4**	92.7
P3	reference polymer, 100% **4**	4.3

suggests that **4** is covalently incorporated into the framework of the imprinted polymer. The relative amounts of **3** extracted in chloroform for **P1** and **P2** are similar to each other and consistent with those reported elsewhere[3e] for L-PheNHPh imprinted polymer extraction in acetonitrile and acetonitrile/acetic acid.[20] It is often argued that the unextractable imprint is located in solvent "inaccessible" regions and can therefore be ignored from the point of view of participating in the imprinted polymer mechanism of molecular recognition.[21] This argument is reasonable if molecular recognition in the imprinted polymer follows Scheme 1. In this configuration, imprint that is unextractable occupies a templated site that could otherwise be used for molecular recognition and renders it inactive because of a lack of void space for adsorption. However, if Scheme 2 controls the molecular recognition in the imprinted material, then a small amount of residual imprint remaining in the polymer can still be significant from the perspective of serving as a nucleation center for adsorption (vide infra). Therefore, Scheme 2 cannot be disqualified on the basis of the amount of imprint extracted from the polymer, unless this number is 100%. In this context, it should be emphasized that not necessarily all of the imprint remaining in the material after extraction serves to act as a nucleation center in Scheme 2. For example, *isolated* unextractable imprint that is adjacent to void spaces surrounded by binding monomer fragments is expected to be more significant in acting as a nucleation center for readsorption in Scheme 2 compared with a clump of imprint and binding monomer fragment that has less void space for readsorption.

Table 2 presents adsorption data for separately adsorbing **3** and its enantiomer onto **P1** and **P2** in chloroform solvent at room temperature.[22] The magnitude of the amount of imprint adsorbed in **P1** and **P2** relative to the maximum number of theoretical sites based on the amount of imprint extracted from the polymer varies from a minimum of approximately 1.06% (D-enantiomer in **P2**) to a maximum of approximately 1.88% (L-enantiomer in **P2**). These values are consistent with those reported for L-PheNHPh imprinted polymer

Table 2. Adsorption of Enantiomers of PheNHPh with Imprinted Polymers[a]

solution	L-PhNHPh solution (μM)	D-PhNHPh solution (μM)	L-PhNHPh adsorbed (μmol/g)	D-PhNHPh adsorbed (μmol/g)
blank	61.9	61.7	0.0	0.0
P1	26.1 ± 1.2	38.5 ± 1.2	3.58 ± 0.12	2.32 ± 0.12
P2	22.0 ± 1.2	39.1 ± 1.2	4.00 ± 0.12	2.27 ± 0.12

[a] Absorbance measurements performed at 260 nm in distilled chloroform. The reported values are the solution and adsorbed concentrations for the various polymers.

systems.[3f] The effect of the 100 ppm addition of **4** is evident. The final solution concentration of **3** is 18.6 ± 12% more for **P1** than for **P2** with the same initial conditions for adsorption (same adsorption conditions). That is to say, **P2** adsorbed 11.5 ± 7% more **3** than did **P1** under identical conditions. Thus, one molecule of **4′** acts as a nucleation center that adsorbs 19 ± 11 molecules of **3** at the conditions used in the adsorption experiment.[23] The data in Table 2 have been reproduced on at least two different synthesis batches of materials and the adsorption experiments repeated approximately a dozen times on each of the materials. (The tolerances reported above represent the peak-to-peak extremities in the observed values of the adsorption experiments.) The trends reported above are also observed at other imprint readsorption concentrations, although here the objective was to characterize materials reproducibly under a single set of experimental conditions.

Summary

The nature of the molecular recognition phenomena in the L-PheNHPh imprinted polymer system has been investigated. Our results show that (i) macroscopic phase separation of **2**−**3** from the remainder of the cross-linked network can occur, (ii) single-crystal X-ray and solution spectroscopic data provide no evidence for multiple-point interactions between **2** and **3**, and (iii) the addition of **4′** into the polymer causes the adsorption of several molecules of **3** per **4′** (should be zero according to Scheme 1). These observations suggest that a mechanism like that schematically illustrated in Scheme 2 could contribute to the enantioselective molecular recognition exhibited by this imprinted polymer system under equilibrium binding conditions. The origin of enantioselective adsorption in Scheme 2 arises from weak interactions between an occluded molecule of **3** (or **4′**) and the molecules of **3** adsorbing from the fluid phases, in a manner described above for the interactions occurring in the crystals of **2**−**3**.[24]

The minimum active fraction of imprint that remains unextractable and participates in this type of adsorption mechanism can be estimated if the ratio of 19 molecules of **3** per **4′** is assumed for molecules of **3** adsorbing per occluded **3**. With polymer **P1**, the calculation yields that only 0.087% of the initial amount of imprint that remains unextractable will be sufficient to account for the adsorption of **3** by **P1**. In other words, the extraction of imprint needs to be greater than 99.9% complete before the mechanism shown in Scheme 2 can be ruled out on the basis of mass balances.

Although one cannot disqualify the possibility that multiple mechanisms can occur in any real material, our results suggest that Scheme 2 is significant in describing the adsorption behavior of the L-PheNHPh imprinted polymer system under the *equilibrium binding conditions* investigated here. In general, we cannot rule out the possibility that a very small number of sites such as those represented in Scheme 1 exist. (They could be below the detection limit of all of the methods used herein.) These sites could provide for selective binding of very low concentrations of adsorbate[3d−g] and for separations by column chromatographic methods.[25] However, even if such is the case, it is clear that one origin for binding site heterogeneity could be that depicted in Scheme 2.

Experimental Section

General Methods. All solvents and chemicals were purchased at the highest grade commercially available. These materials were not purified further except when used for the synthesis of the imprinted polymers. Solvents for the imprinted polymer syntheses were prepared as follows. Chloroform (EM Science Omnisolve Grade) was washed with distilled water three times followed by washings with saturated sodium bicarbonate and brine solutions. The chloroform was dried with calcium chloride for a period of 12 h and subsequently dried with phosphorus pentoxide for a period of 24 h. The chloroform was then distilled under positive argon pressure from the phosphorus pentoxide. Ethylene glycol dimethacrylate (Aldrich) was washed three times with 15% aqueous NaOH followed by washings with saturated sodium bicarbonate and brine solutions. The ethylene glycol dimethacrylate was dried with calcium chloride for a period of 12 h and subsequently dried with calcium hydride for a period of 24 h. It was then degassed by sonication and distilled under a vacuum of 300 mTorr from calcium hydride. Methacrylic acid (Aldrich) was dried for 24 h with calcium chloride and distilled from the calcium chloride under a vacuum of 1 Torr prior to use for the imprinted polymer syntheses. All ^1H and ^{13}C NMR spectra were recorded using a General Electric QE 300 Plus spectrometer operating at 300.3 and 75.5 MHz, respectively. Reported chemical shifts are relative to the central peak of the CHD_2CN heptet at 1.930 ppm (relative to TMS) for deuterated acetonitrile solvent or the $CHCl_3$ singlet at 7.240 ppm (relative to TMS) for deuterated chloroform. Liquid-phase ^{19}F NMR spectra were recorded at 282.203 MHz in ^{19}F-labeled CCl_3F solvent using a Varian 300 MHz spectrometer. Weight measurements were performed using a Mettler AE 260 Deltarange balance (one-tenth of a milligram resolution). Room temperature was controlled to be 24 ± 1 °C unless otherwise specified.

Synthesis of L-Phenylalanine Anilide. To a mixture of 105 mmol of N-*t*-BOC-phenylalanine (28 g) and 103 mmol of hydroxybenzotriazole (15.7 g) in 320 mL of CH_2Cl_2 was added 116 mmol of dicyclohexylcarbodiimide (24 g) over ice cooling. Subsequently 116 mmol of aniline (10.6 mL) was added to the reaction mixture over ice cooling The reaction mixture was stirred at room temperature and monitored by TLC using 9:1 chloroform/methanol as eluent and Silica Gel 60 F_{254} plates (EM Science). The insoluble dicyclohexylurea was filtered, and the crude solution was redissolved in $CHCl_3$, washed with 0.1 M HCl/saturated sodium bicarbonate/brine solutions, and dried over magnesium sulfate. The resulting solution was evaporated to a viscous oil and eluted through a silica gel column packed with Silica Gel 60 (EM Science) using 2.5:1 hexane:ethyl acetate as eluent. The crude product comprising N-*t*-BOC-phenylalanine anilide was recrystallized in chloroform/hexane at room temperature to give 88 mmol (30 g) of transparent platelet crystals of N-*t*-BOC-phenylalanine anilide (yield 84%). Deprotection was initiated by adding neat trifluoroacetic acid under ice cooling to the crystals. The deprotection reaction was conducted at room temperature and monitored by a bubbler. When gas evolution had completely stopped, the trifluoroacetic acid was evaporated to a viscous transparent oil. The oil was made basic and was extracted repeatedly with chloroform, washed with distilled water/saturated sodium bicarbonate/brine solutions, and dried over potassium carbonate. Transparent platelets of the target compound were recrystallized from chloroform/hexane to give 62 mmol (14 g) of product (yield 59%); mp 72 °C (uncorrected) [lit.[26] 71−72 °C]; $[\alpha]^{22}_{D}$ −136.7° (c 0.3, $CHCl_3$) [lit.[27] $[\alpha]^{20}_{D}$ +137 (c 0.3, $CHCl_3$) for D-phenylalanine anilide]. ^1H NMR ($CDCl_3$): 1.55 (2H, s, NH_2); 2.77 (1H, dd, J = 9.6 Hz, 13.8 Hz, CHH); 3.38 (1H, dd, J = 3.9 Hz, 13.8 Hz, CHH); 3.73 (1H, dd, J = 3.9 Hz, 9.6 Hz, CH); 7.08−7.62 (10H, m, Ph-H); 9.45 (1H, brs, CONH). $^{13}C\{^1H\}$ NMR ($CDCl_3$): 40.8 (Ph−CH_2); 56.9 (CHCO); 119.5 (CH); 124.2 (CH); 127.0 (CH); 128.9 (CH); 129.0 (CH); 129.4 (CH); 137.8 (C_q); 172.5 (C=O). Anal. Calcd for $C_{15}H_{16}N_2O$: C, 74.97; H, 6.71; N, 11.66. Found: C, 74.84; H, 6.85; N, 11.65.

Synthesis of L-Phenylalanine 4-Vinylanilide. To a mixture of 77 mmol of N-FMOC-L-phenylalanine (30 g) and 83 mmol of 4-vinylaniline (9.75 mL) in 250 mL of THF under ice cooling and an argon atmosphere was added 80 mmol of diisopropylcarbodiimide (12.5 mL). The reaction mixture was stirred for approximately 3 h at room temperature. Upon filtering the insoluble urea and evaporating the solvent, a crude product was crystallized from 500 mL of methanol. The crystals were filtered and washed with methanol. A 68 mmol (33.2 g) sample of N-FMOC-L-phenylalanine vinylanilide was isolated (yield 88%). Deprotection was performed by treating the crystals with a 5 vol % piperidine solution in THF overnight at room temperature. The THF was evaporated, and the resulting crude flocculent precipitate was dissolved in ethyl acetate, evaporated to an oily residue, and purified through a silica gel column packed with Silica Gel 60 using a gradient solvent mixture starting with 3−5% ethyl acetate in CH_2Cl_2 and ramping to 70% ethyl acetate in CH_2Cl_2 once the FMOC−piperidine complex was removed from the column as monitored by TLC using 8:2 CH_2Cl_2/ethyl acetate as eluent and Silica Gel 60 F_{254} plates. Transparent platelets of the desired L-phenylalanine vinylanilide product were recrystallized from chloroform/hexane to give 43 mmol (11 g) of product (yield 56%); mp 75 °C (uncorrected) [lit.[17] 79 °C]; $[\alpha]^{22}_{577}$ −101.7° (c 0.87, acetone). Enantiomeric purity was evaluated by derivatizing the final product with the NMR resolving agent S-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid; a single peak in the liquid-phase ^{19}F NMR spectrum of the derivitized product was obtained.[28] ^1H NMR ($CDCl_3$): 1.53 (2H, s, NH_2); 2.79 (1H, dd, J = 9.6 Hz, 13.8 Hz, CHH); 3.39 (1H, dd, J = 3.8 Hz, 13.8 Hz, CHH); 3.73 (1H, dd, J = 3.9 Hz, 9.6 Hz, CH); 5.22 (1H, d, J = 10.9 Hz, vinyl CHH); 5.72 (1H, d, J = 17.6 Hz, vinyl CHH); 6.70 (1H, dd, J = 10.9 Hz, 17.6 Hz); 7.25−7.61 (9H, m, Ar-H); 9.52 (1H, s, CONH). $^{13}C\{^1H\}$ NMR ($CDCl_3$): 40.8 (Ph−CH_2); 56.9 (CHCO); 113.0 (vinyl CHH); 119.5 (CH); 126.9 (CH); 127.0 (CH); 128.9 (CH); 129.4 (CH); 133.6 (C_q); 136.3 (CH); 137.4 (C_q); 137.8 (C_q); 172.5 (C=O). Anal. Calcd for $C_{17}H_{18}N_2O$: C, 76.67 H, 6.81; N, 10.52. Found: C, 76.43; H, 6.69; N, 10.49.

Titration Experiments via ^1H NMR and FTIR Spectroscopies. The samples for the titration studies were individually prepared in scintillation vials by weighing out portions of l-phenylalanine anilide and methacrylic acid to correspond to the desired number of equivalents for the particular sample being prepared. Chloroform or acetonitrile (deuterated for NMR) was then added so as to maintain an overall concentration of L-phenylalanine anilide of 0.1 M in the final solutions. The samples were then degassed for 15 min in a sonicator prior to analysis. For the titration experiments measured by ^1H NMR, all spectra were recorded at temperatures of 23, 35, 45, and 60 °C within a deviation of 0.2 °C. For the case of the FTIR titration, all spectra were recorded at room temperature on a Nicolet System 800 FTIR spectrometer with a 0.1 mm path length, CaF_2 solution phase cell (Spectra Tech FT04-757) by averaging 256 scans.

X-ray Structure Determination Crystal Growth. A 10 mL aliquot of a 0.2 M solution of l-phenylalanine anilide in $CHCl_3$ was placed in a 20 mL scintillation vial, and 4 mol equiv of methacrylic acid (0.678 mL) was added. The open vial was then placed in a covered cylindrical TLC tank (10 × 20 cm) that was filled partially with hexane and carefully held there at room temperature. After a period of 3 days long needles were observed optically in the solution. A portion of these needles was extracted with tweezers under a polarizing microscope and used as specimens for the X-ray structure solution.

X-ray Structure Determination. Data were collected at room temperature on a Syntex (Crystal Logic) diffractometer with Cu Kα X-rays. The structure was solved by direct methods and refined to a final agreement factor of $R = 5.8\%$ and $R_w = 6.0\%$ for 803 reflections with $F > 2\sigma(F)$. Calculated hydrogen positions were included in the structural analysis.

P1 Imprinted Polymer Reaction Mixture. A previously reported recipe was followed for all imprinted polymer syntheses.[3g] In a 38 mL pressure tube (Ace Glass 8648-29), 0.7209 g of l-phenylalanine anilide imprint and 5.1 mL of $CHCl_3$ were combined. Upon complete dissolution of the imprint, 1.017 mL of methacrylic acid and 11.32 mL of ethylene glycol dimethacrylate were added. To this mixture was added 5.25 mL of $CHCl_3$ containing 0.1232 g of azobis(isobutyronitrile). Finally 6 mL of $CHCl_3$ was added. The mixture was sealed with a septum for further processing.

P2 Imprinted Polymer Reaction Mixture. In a 38 mL pressure tube (Ace Glass 8648-29), 0.4806 g of L-phenylalanine anilide imprint and 4.4 mL of $CHCl_3$ were combined. Upon complete dissolution of the imprint, 0.678 mL of methacrylic acid and 7.54 mL of ethylene glycol dimethacrylate were added. To this mixture was added 3.5 mL of $CHCl_3$ containing 0.0821 g of azobis(isobutyronitrile). A 2 mL aliquot of $CHCl_3$ was added. Finally 1 mL of a 0.2 mM solution (2×10^{-7} mol total) of L-phenylalanine 4-vinylanilide in $CHCl_3$ was added to the reaction mixture, and the mixture was sealed with a septum for further processing.

P3 Imprinted Polymer Reaction Mixture. In a 38 mL pressure tube (Ace Glass 8648-29), 0.5327 g of L-phenylalanine 4-vinylanilide imprint and 5.0 mL of $CHCl_3$ were combined. Upon complete dissolution of the imprint, 0.678 mL of methacrylic acid and 7.54 mL of ethylene glycol dimethacrylate were added in the order listed. To this mixture was added 3.5 mL of $CHCl_3$ containing 0.0821 g of azobis(isobutyronitrile). Finally 2.4 mL of $CHCl_3$ was added. The mixture was sealed with a septum for further processing.

Imprinted Polymer Synthesis and Extraction. The sealed pressure tubes were freeze−thaw−degassed by submerging the tubes in liquid nitrogen and holding the frozen tubes under a vacuum of 100 mTorr for a period of at least 15 min for each cycle. Each sample was freeze−thaw−degassed in this manner for three cycles. The tubes were then thawed and charged with a positive pressure of argon. The tubes were placed in ice-filled dewars and temperature equilibrated. These dewars were then placed under a water-cooled medium-pressure mercury vapor lamp (Conrad Hanovia 550 W) for a period of 24 h. During this time the tubes were turned, and the ice in the dewars changed three times to maintain a constant temperature of 0 °C during the entire polymerization.[29] Upon completion of the polymerization, the tubes were taken out of the dewars and crushed. The polymers were ground in a ball mill (Spex 8000) and dry sieved (Spex 3536) to a size between 100 mesh (149 μm) and 200 mesh (74 μm). The sieved polymer particles were dried at room temperature for a period of 24 h in a 10 mL airless flask connected to a vacuum manifold to remove residual solvent (40 mTorr). The weighed polymers were placed in a cellulose extraction thimble (Whatman 2800199) and Soxhlet extracted in $CHCl_3$ for a period of 24 h.[30] The extracted polymers were dried at room temperature for a period of 24 h in a 10 mL airless flask on a vacuum manifold (40 mTorr). The extract was filtered directly into a clean scintillation vial using a 0.7 μm pore size GF/F Filter Media syringe filters (Whatman 6825-1307). The extract was diluted, and its UV absorbance value was recorded at a fixed wavelength of 260.0 nm and a 1 s integration time using a Beckman DU-640 spectrophotometer and blackened standard size UV cells (Starna 29BQ10). The amount extracted was calculated directly from this absorbance value using a theoretical maximum amount extracted of 0.2179 mmol imprint per gram of solvent-free as-synthesized polymer and ϵ(260 nm) = 8523 M^{-1}. (For **P3** the theoretical maximum amount is 0.2166 mmol imprint per gram of solvent-free as-synthesized polymer, and ϵ(260 nm) = 15 083 M^{-1}.)

Liquid-Phase Dye Visualization Procedures. In a disposable culture tube, 0.0961 g of l-phenylalanine anilide imprint, 0.4 mg of chlorophenol red dye (Sigma), and 0.5 mL of $CHCl_3$ were combined. Upon complete dissolution of the imprint, 136 μL of methacrylic acid and 1.5 mL of ethylene glycol dimethacrylate were added. To this mixture was added 0.7 mL of $CHCl_3$ containing 16 mg of azobis(isobutyronitrile). Finally 0.98 mL of $CHCl_3$ was added.

Batch Adsorption Experiments with Imprinted Polymers. Into a short form 1 dram (4 mL) vial covered with a Teflon cap (Qorpak 5200) were added 40 mg of imprinted polymer and 4.0 mL of D- or L-phenylalanine anilide at a concentration of 6.18×10^{-5} M in $CHCl_3$ (target absorbance value of 0.5265 in $CHCl_3$ since $\epsilon(260\ \text{nm}) = 8523\ M^{-1}$). The vial was covered, and the mixture in the vial was stirred using a flea micro stirring bar (Bel-Art) at room temperature. The solutions in the vials were equilibrated for a period of 24 h at these conditions. Afterward, the contents of the vial were filtered directly into a UV cuvette using a 0.7 μm pore size GF/F Filter Media syringe filters (Whatman 6825-1307). All UV measurements were performed at a fixed wavelength of 260.0 nm and a 1 s integration time using a Beckman DU-640 spectrophotometer and blackened standard size UV cells (Starna 29BQ10).

Acknowledgment. A.K. acknowledges a John and Fannie Hertz Foundation Graduate Fellowship. The authors thank Dr. Lech Dudycz (Brandeis University) for assistance in the synthesis of compound **4** and Dr. Saeed Khan (University of California Los Angeles) for the structure solution of the crystal of **2** and **3**. Helpful discussions with Dr. Hector Gonzalez, Professor Robert H. Grubbs, and Professor Zhen-Gang Wang at Caltech are gratefully acknowledged. A.K. thanks the technical assistance of Mr. Taison Tan.

Supporting Information Available: Tables of crystallographic data for the **2**–**3** crystal and figures showing plots of shift of exchangeable proton of **3** vs molar equivalents of **2**, IR spectra of **2** and **3** in chloroform solution, and optical photographs of **P1** and **P2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) For discussion relating to nucleation centers formed from residual imprint in an imprinted polymer system, see: (a) Dickey, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **1949**, *35*, 227. (b) Dickey, F. H. *J. Phys. Chem.* **1955**, *59*, 9, 695. (c) Morrison, J. L.; Worsley, M.; Shaw, D. R.; Hodgson, G. W. *Can. J. Chem.* **1959**, *37*, 1986. The ability of such nucleation centers that comprise a chiral amino acid derivative attached to a material surface to provide for chiral molecular has been demonstrated previously, as, for example, in: Frank, H.; Nicholson, G. J.; Bayer, E. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 363.

(9) A minimum of three independent binding interactions are required for enantioselective molecular recognition (see: Ogsten, A. G. *Nature* **1948**, *162*, 963). For discussion related to a more modern application, see: Arena, G.; Bonomo, R.; Casella, L.; Gullotti, M.; Impellizzeri, G.; Maccarrone, G.; Rizzarelli, E. *J. Chem. Soc., Dalton Trans.* **1991**, 3203 and discussion therein.

(10) There is literature precedent for infrared characterization of multiple-point binding interactions. For example, infrared shifts greater than 20 cm^{-1} have been observed for the N–H stretching vibrations corresponding to each of the three functional groups involved in the hydrogen bonding between the adenine and uracil derivatives 2,6-diamino-9-ethylpurine and 1-cyclohexyl-5-bromouracil, respectively (Kyogoku, Y.; Lord, R. C.; Rich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *57*, 250).

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(12) Liquid-phase ^{13}C NMR in deuterated chloroform was used to corroborate the FTIR results. To avoid accidental overlap of the carbonyl carbon resonances between the amide **3** and the acid **2**, a titration experiment was performed with the structurally similar propionic acid and **3** using the same experimental conditions described in the paper for the FTIR titration. It has been previously established that a hydrogen bond involving the C═O functional group shifts the carbonyl carbon resonance downfield (Kalinowski, H.; Berger, S.; Braun, S. *Carbon-13 NMR Spectroscopy*; John Wiley & Sons: New York, 1988). No downfield shift in the amide carbonyl carbon resonance of **3** was observed during the course of titrating up to 4 mol equiv of propionic acid.

(13) Crystallization within the imprinted polymer of the same crystals as reported for chloroform was also observed with more polar solvents such as acetonitrile and at higher temperatures of up to 80 °C. ^1H NMR of the crystals isolated from the polymer was performed under the same conditions as discussed for the NMR acid titration experiment (the crystal was dissolved at the same concentration of **3** in deuterated chloroform). Integration of the exchangeable proton yielded approximately three protons indicative of a 1:1 molar ratio of **2** and **3** in the crystal. By measuring the chemical shift of the exchangeable proton at 23 °C to be 5.24 ppm under the same conditions as in Figure 4a (see Supplementary Information where 5.27 ppm is predicted from the NMR acid titration experiment in deuterated chloroform), it was determined that the crystals comprised a 1:1 molar ratio of **2** and **3**.

(14) In an attempt to resolve any potential differences between crystals grown in the polymer during material synthesis and crystals grown for the single crystal study as described in the paper, X-ray powder diffraction on the single crystals and the collected material from the polymer synthesis was performed. The overlap in the X-ray powder diffraction patterns indicates that the material formed under polymerization conditions is indeed structurally the same with that synthesized under more careful conditions to yield larger single crystals.

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(18) A crystal **C1** was synthesized from a chloroform solution containing initially 70 mol % **3** and 30 mol % **4** as the amino acid derivative content and 4 mol equiv of methacrylic acid by using the same procedures for crystal growth as described in the Experimental Section. Most major reflections in the X-ray powder pattern of this crystal overlap with the crystal synthesized from **3** alone, suggesting that the species comprising both crystals are similar in their orientation and positioning. On the basis of the integration of the vinyl proton resonances of the ^1H NMR of dissolved crystal **C1** in deuterated chloroform, it was estimated that the content of **4** in

the crystal **C1** was approximately 21 mol %, indicating 70 mol % incorporation of **4** from the mother liquor into the crystal. A single amine proton resonance was observed in the ^{1}H NMR of dissolved crystal **C1** in deuterated chloroform, which is consistent with the similarity of **4** and **3** in interacting with **2** in the solution phase.

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(20) Further Soxhlet extraction of **P1** in a mixture of 7:3 MeOH: acetic acid (v:v) for 24 h and chloroform for 36 h resulted in a polymer posessing an imprint adsorption capacity differing by less than 1% relative to **P1**. Furthermore, no increase in the enantioselectivity was observed by comparison to **P1** upon imprint rebinding. Confocal fluoresence microscopy (Biorad 600 confocal fluoresence microscope using excitation wavelength of 568 nm and emission wavelength of long-pass filter 50% level at 587 nm) was used to observe occluded imprint following extraction. An imprinted polymer was synthesized and processed for this purpose according to the same experimental procedure as for **P1** only using L-phenylalanine β-naphthylamide (Sigma) as the imprint. The faint fluorescence observed in the extracted polymer signifies that some imprint still remains in the polymer after extraction.

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(23) Assuming an average of 93% imprint extraction from the imprinted polymers, one obtains that there are 8.5196×10^{-3} mmol of theoretical binding sites per 40 mg of extracted imprinted polymer (based on the amount of imprint originally in the polymer prior to extraction). **P1** adsorbed the equivalent 1.68% of the theoretical maximum of sites with **3** while **P2** adsorbed the equivalent of 1.88% of the theoretical maximum of sites with **3**. Calculating the difference between these two polymers and dividing it by the fraction of **4** in **P2** (0.01%) gives 19 ± 11 molecules of **3** adsorbed per fragment of **4**.

(24) The mechanism represented by Scheme 2 affords a nonlinear adsorption isotherm, as the environment an adsorbing molecule experiences in such a case is strongly dependent on the number of molecules already adsorbed adjacent to the binding site in Scheme 2.

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(30) Prior to extracting any of the polymers, the rates of mass transfer under the adsorption conditions used (at room temperature) were investigated. The time to achieve mass-transfer-limited equilibration in a L-PheNHPh imprinted polymer (prepared identically to the procedure given for **P1**) was less than 10 min for both the L- and D-enantiomers of **3** (with the particle sizes used in this work).

MA981445Z