Surface Imprinted Polymers Recognizing Amino Acid Chirality

MASAHIRO YOSHIDA, KAZUYA UEZU, MASAHIRO GOTO, SHINTARO FURUSAKI

Department of Chemical Systems and Engineering, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan

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ABSTRACT: A highly enantioselective polymer was prepared for the separation of optically active tryptophan methylester by a surface molecular imprinting technique. An organophosphorus compound was found to be effective as a functional host molecule for recognizing the chirality of the amino acid ester. The imprinted polymers exhibited a higher template effect toward the corresponding imprinted tryptophan methylester than its isomer and analogues, while a reference polymer prepared without an imprinting molecule did not show any selectivity toward the enantiomers. The enantioselective recognition was quantitatively evaluated by determination of the binding constants of the D- and L-tryptophan methylester to the imprinted polymers. Furthermore, the mechanism for producing enantioselectivity was deduced from FTIR and ^{1}H-NMR spectra. Based on the results obtained we concluded that the enantiomeric selectivity was mainly caused by electrostatic and hydrogen bonding interactions between the functional organophosphorus molecule and the target tryptophan methylester on the polymer surface. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 78: 695–703, 2000

Key words: molecular imprinting; surface molecular imprinting; enantioselectivity; amino acid; adsorption

INTRODUCTION

The creation of artificial receptors that can accomplish recognition at the molecular level is one of the major goals in chemistry. To date, various synthetic molecular clefts and cavities (e.g., crown ethers, cyclodextrins, and cycrophanes) have been developed by utilizing the basic or specific interactions such as hydrogen bonding, ionic interaction, van der Waals interaction, hydrophobic effect, and so forth. Novel receptors are emerging rapidly.[1–5]

Another promising approach to design synthetic receptor-like binding sites is the molecular imprinting technique.[6–8] Polymers prepared by this technique have attracted much attention as interesting separation tools, especially for high-performance liquid chromatography (HPLC). Important applications are optical resolutions of amino acids or amino acid derivatives,[9–15] direct enantioseparation of drugs such as β-adrenergic blockers,[16,17] and regio- and enantioseparation of sugar or sugar derivatives.[18,19] In addition, characteristic antibodies or enzyme analogues prepared by the molecular imprinting technique have been studied extensively.[20–25]

The imprinting technique is conceptually easy to apply to a wide variety of target molecules. However, it still has some fundamental drawbacks that remain unresolved, such as the inapplicability to water-soluble substances, which are important in the biological or biomedical field,

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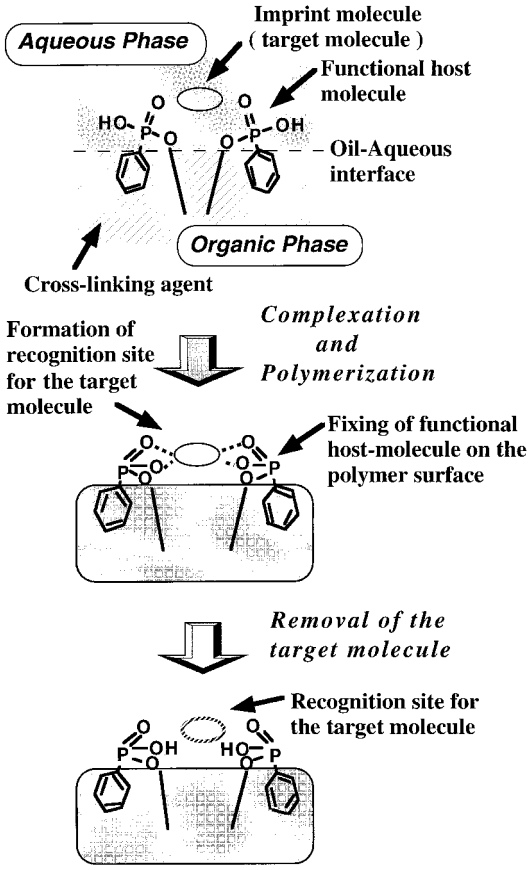

Figure 1 Schematic illustration of the surface molecular imprinting technique.

and the slow rebinding kinetics arising from the inner diffusion of imprint molecules toward the recognition sites that are formed deeply inside the polymer matrix.

Recently, we proposed a novel molecular imprinting technique to overcome these problems, the "surface molecular imprinting technique."[26–32] The general idea of the surface molecular imprinting technique is illustrated in Figure 1. The solid polymer, which is molecularly imprinted at the internal cavity surface, is prepared by polymerizing water in oil (W/O) emulsions consisting of a water-soluble imprint molecule, a functional host molecule to interact with the imprint molecule, an emulsion stabilizer, and a crosslinking agent. In this novel technique the organic–aqueous interface in W/O emulsions is utilized as the recognition field for a target molecule. The target molecule forms a complex with the functional host molecule, while the orientation of the functional host molecule itself is fixed at the oil–water interface. After polymerization this provides the recognition sites at the inner cavity surface of the imprinted bulk polymer. The complex between the functional host molecule and the target material should not be too hydrophobic or hydrophilic, because otherwise the complex would not be located at the oil–water interface. Thus, a functional host molecule should be amphiphilic just like a surfactant molecule in order to yield a high template effect for the target molecule. The crosslinking agent crosslinks the organic phase and stabilizes the water pool or the imprinted water cavity after polymerization. The bulk polymer obtained is ground to small particles in order to interact with the target molecules in the solution. This technique also realizes a rapid and reversible complexation of target molecules with the imprinted polymer.

From the viewpoint of the preparation of an effective imprinted polymer, in our previous studies we clarified that there are three essentials needed by the functional host molecule to obtain a high template effect: a long alkyl chain that yields a high interfacial activity on the organic–aqueous phase, an aromatic ring to enhance the rigidity of the recognition sites, and a target recognition organophosphorus group that can produce a high binding affinity with the target molecule.[30–32] To fulfill the above conditions we designed a novel functional host molecule, phenyl phosphonic acid monododecyl ester (*n*-DDP), which consists of a 12 methylene chain, an aromatic ring, and a phosphonic acid group. In our previous study we demonstrated that a zinc ion imprinted polymer that includes *n*-DDP exhibits significant high selectivity toward zinc ions over copper ions in a competitive zinc–copper sorption test.[31]

In the present article we demonstrate an enantioselective template effect at the surface of the imprinted polymers. We used *n*-DDP as a functional host monomer and chose D, L-tryptophan methylester (D,L-TrpOMe) as the target molecule. The enantioselective performance of the D- or L-TrpOMe imprinted polymer was evaluated by a competitive adsorption test. The adsorption behavior of D,L-phenylalanine OMe (D,L-PheOMe) and D,L-Trp, which are analogues of the target TrpOMe, were also investigated to identify the substrate specificity. The template effect was also characterized by comparing the imprinted polymers with those of the unimprinted adsorbent. Furthermore, we quantitatively discuss the template effect in regard to the D- or L-TrpOMe imprinted polymer by evaluating its binding constant on the basis of a modified Scatchard analy-

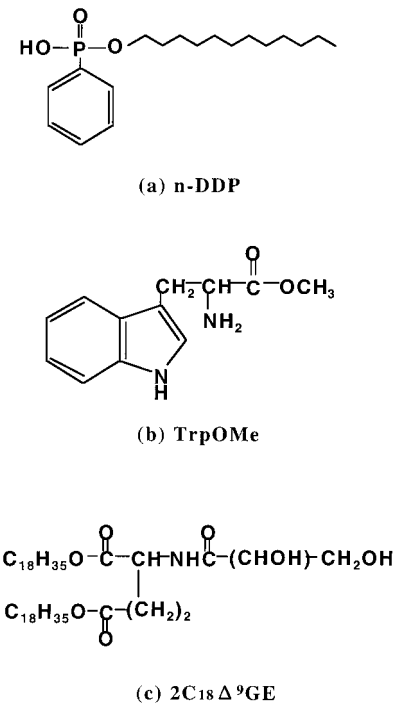

Figure 2 Structures of (a) the functional host molecule *n*-DDP, (b) the imprint molecule TrpOMe, and (c) the emulsion stabilizer $2C_{18}\Delta^9GE$.

sis.[33,34] The specific interactions between TrpOMe and the functional host molecule *n*-DDP at the prepolymerization stage were investigated in detail by utilizing FTIR and ^{1}H-NMR spectra to elucidate the imprinting phenomena and the enantioselectivity toward TrpOMe.

EXPERIMENTAL

Reagents and Apparatus

The *n*-DDP and L-glutamic acid dioleylester ribitol ($2C_{18}\Delta^9GE$) were synthesized according to the procedures reported in previous works.[31,35] The D,L-TrpOMe, D,L-PheOMe, and D,L-Trp were purchased from Sigma Chemical Co. Divinylbenzene (DVB, Wako Pure Chemical Industries Co., Ltd.) was employed after treatment with silica gel to remove an inhibitor. Figure 2 shows the structures of *n*-DDP, TrpOMe, and $2C_{18}\Delta^9GE$. Other reagents were of commercially available grades. Particle size analysis was performed by a microtrac optical analyzer (model 7995-10 SRA, Nikkiso Co., Ltd.), and the electron scanning was carried out using an ABT-32 type microscope (Akashi Beam Technology Co., Ltd.). The surface area of the polymers was obtained by performing the N_2 adsorption test with a micrometrics ASAP 2000 instrument (Shimadzu).

Preparation of D- or L-TrpOMe Imprinted Polymer

Enantioselective polymers were prepared by the surface molecular imprinting technique utilizing W/O emulsions. A 40-mL amount of DVB containing 60 mol/m^3 *n*-DDP and 5 mol/m^3 $2C_{18}\Delta^9GE$ was mixed with 20 mL of toluene. A 30-mL aqueous solution containing 10 mol/m^3 D- or L-TrpOMe (pH adjusted to 4.5 with 100 mol/m^3 phosphate buffer solution) was then added to the above organic phase. The mixture was sonicated for 4 min to obtain stable W/O emulsions. After the addition of 0.36 g (1.4×10^{-3} mol) of powder initiator [2,2′-azobis(2,4′-dimethylvaleronitrile), 0.01 wt % for DVB], the mixture was polymerized at 55°C for 2 h under a flow of nitrogen. The obtained bulk polymer was dried under a vacuum and ground into particles of an appropriate size. The particles were washed with 1000 mol/m^3 hydrochloric acid to remove the imprinted D- or L-TrpOMe and then filtered off. This procedure was repeated several times until the imprint molecule in the filtrate could not be detected by a UV spectrometer. Finally, the polymer was dried in vacuo for several days. As reference, an unimprinted polymer was similarly prepared without the imprinting molecule.

Competitive Adsorption Experiments on D- or L-TrpOMe Imprinted and Unimprinted Polymers

The batchwise selective adsorption experiments of D,L-TrpOMe, D,L-PheOMe, and D,L-Trp were conducted for the D- or L-TrpOMe imprinted and unimprinted polymers. The polymers (0.05 g) were added to 5-mL of aqueous solution containing 0.5 mol/m^3 D- or L-TrpOMe and placed in a sealed test tube (10-mL volume). The pH was adjusted to a desired value of between 1.5 and 7.5 with 100 mol/m^3 KH_2PO_4—K_2HPO_4 and 100 mol/m^3 HNO_3. The mixture was shaken in a thermostated water bath at 30°C for 24 h. The polymers were then filtered off through a polyethylene membrane (Sumplep LCR25-LG, Nippon Millipore, Ltd.). The amount of each amino acid derivative adsorbed to the polymers was calculated from their residual amount in the filtrate. The concentration of amino acid derivatives was analyzed by an HPLC system (Japan Spectroscopic Co., Ltd.) with a reversed phase column

(TSKgel ODS-80T_s column, 4.6 × 250 mm, Toyo Soda Co., Ltd.). The elution [100 mol/m^3 CH_3COOH—CH_3COONa (pH 4.5)/acetonitrile = 4/1 (v/v)] was spectrophotometrically monitored at 278.8 nm and 1.0 mL/min except for PheOMe, whose elution was monitored at 258 nm and 0.5 mL/min. The adsorption tests were conducted at least 3 times and the data were plotted with the average values. The experimental errors were less than 8%.

Binding Constants of D- or L-TrpOMe for Imprinted Polymer

Binding constants of D,L-TrpOMe on the D- or L-TrpOMe imprinted polymer were evaluated with the batchwise method. A 0.05-g polymer sample was immersed in a sealed test tube (10-mL volume). Then a 5-mL aqueous solution was added, which was buffered with 100 mol/m^3 KH_2PO_4—K_2HPO_4 containing D- or L-TrpOMe adjusted to a desired concentration of between 0.05 and 5 mol/m^3. The mixture was shaken at room temperature for 24 h. The polymers were then filtered off through the polyethylene membrane. The concentration of D- or L-TrpOMe in the filtrate was analyzed by means of the HPLC system. The binding constants were calculated by a modified Scatchard equation.

FTIR and ^{1}H-NMR Studies

FTIR and ^{1}H-NMR spectra studies were carried out using an FTIR 8300 (Shimadzu) and an AC 250 P (Bruker) at 25°C. ^{1}H-NMR samples were measured in C_6D_6 containing 15 vol % DMSO-d_6 (250 MHz, TMS as internal standard).

RESULTS AND DISCUSSIONS

Physicochemical Characterization of D- or L-TrpOMe Imprinted and Unimprinted Polymers

Highly crosslinked D- or L-imprinted and unimprinted polymers were prepared by the surface molecular imprinting technique with W/O emulsions. D- or L-TrpOMe, *n*-DDP, toluene, DVB, and $2C_{18}\Delta^9GE$ were used as an imprint molecule, a functional host molecule, a diluent, a crosslinking agent, and an emulsion stabilizer, respectively. The D- or L-TrpOMe imprinted and unimprinted polymers were obtained at more than 80% yields. After polymerization the bulk D- or L-TrpOMe imprinted and unimprinted polymers were ground into particles whose volume-averaged diameters were about 40 μm in all experiments. Figure 3 shows a typical view of the TrpOMe-imprinted polymers prepared from W/O emulsions by scanning electron microscopy. For polymers utilizing W/O emulsions, substantial traces of aqueous phases in the emulsions are observed in the polymer. The recognition sites for D- or L-TrpOMe are constructed on the surfaces of the inner cavities of the polymer. A number of micropores in the polymers facilitate diffusion of the target TrpOMe into the polymer. According to the N_2 adsorption test, the surface area of the imprinted polymer was found to be 15.1 m^2/g polymer. The particle was employed in the subsequent adsorption tests.

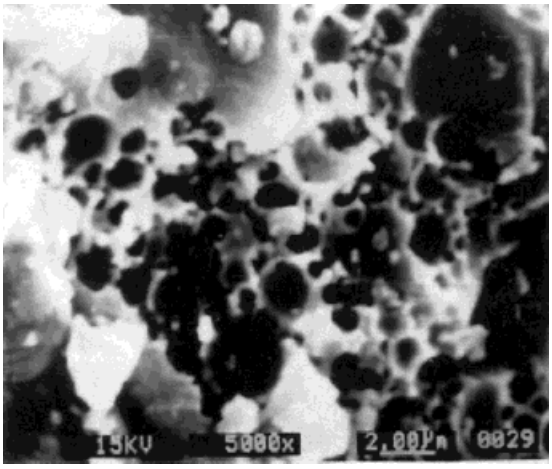

Figure 3 A typical scanning electron microscopy photograph on the TrpOMe imprinted polymers.

Adsorption Behavior of D- and L-TrpOMe Imprinted and Unimprinted Polymers

Figures 4 and 5 exhibit the pH dependence of the adsorption of D,L-TrpOMe, D,L-PheOMe, and D,L-Trp on the L-TrpOMe imprinted polymer. The percentage of adsorption increased with increasing the pH of the material solution. This result means that the proton dissociation of the functional host molecules implanted on the polymer surface plays a predominant role in the binding of the amino acid derivatives. The L-TrpOMe imprinted polymer showed a high template effect toward L-TrpOMe over D-TrpOMe in the whole pH range studied (Fig. 4). The adsorption behavior of PheOMe exhibited no selectivity on the L-TrpOMe imprinted polymer (Fig. 5). This result indicates the importance of the indole ring for producing

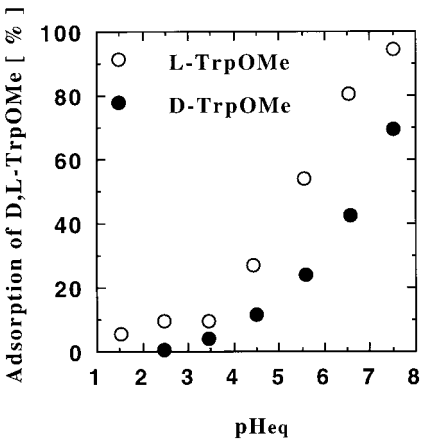

Figure 4 The pH dependence of the adsorption of D- and L-TrpOMe on the L-TrpOMe imprinted polymer.

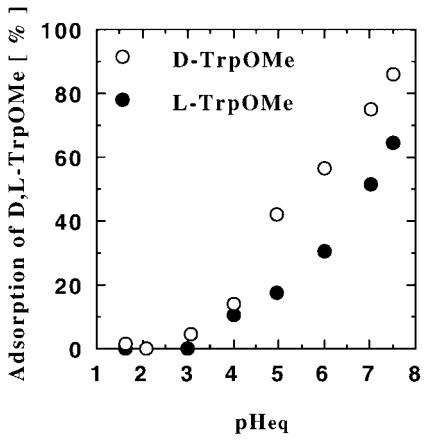

Figure 6 The pH dependence of the adsorption of D- and L-TrpOMe on the D-TrpOMe imprinted polymer.

the enantioselectivity. Furthermore, tryptophan was not adsorbed on the L-TrpOMe imprinted polymer at all (Fig. 5). On the other hand, the D-TrpOMe imprinted polymer showed a high template effect toward the imprinted D-TrpOMe molecule (Fig. 6). The adsorption behavior of D,L-PheOMe and D,L-Trp on the D-TrpOMe imprinted polymer was similar to that observed in the L-TrpOMe imprinted polymer (Fig. 7). The strong binding of PheOMe to the TrpOMe imprinted polymer was caused by the nonspecific electrostatic interaction. The number of nonspecific adsorption sites for PheOMe is considered to be more than that of organized recognition sites for TrpOMe. In addition, although the unimprinted polymer prepared without the imprint molecule has a high potential for adsorption, it afforded no evidence of enantioselective adsorption (Figs. 8, 9). This was probably caused by the random distribution of the functional host molecules on the polymer surface.

Amino acids (D- and L-Trp) were neither adsorbed on the D- and L-TrpOMe imprinted polymers nor on the unimprinted polymers (Figs. 5, 7, 9), because amino acids form intramolecular ionic complexes between amino and carboxyl groups in the molecules. This result means that the electrostatic interaction between the phosphonic acid moiety in the functional host molecule and the amino group in the substrate is one of the vital factors for creating the enantiomeric selectivity. Furthermore, no enantioselectivity of D and L-PheOMe on the D- or L-TrpOMe imprinted polymer suggests the crucial effect of the indole ring

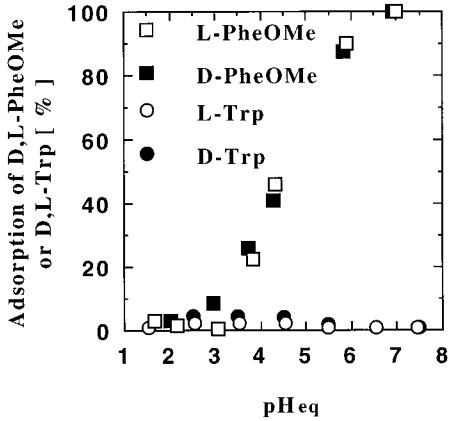

Figure 5 The pH dependence of the adsorption of D- and L-PheOMe or D- and L-Trp on the L-TrpOMe imprinted polymer.

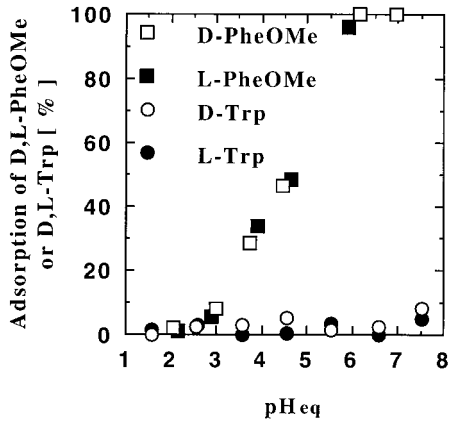

Figure 7 The pH dependence of the adsorption of D- and L-PheOMe or D- and L-Trp on the D-TrpOMe imprinted polymer.

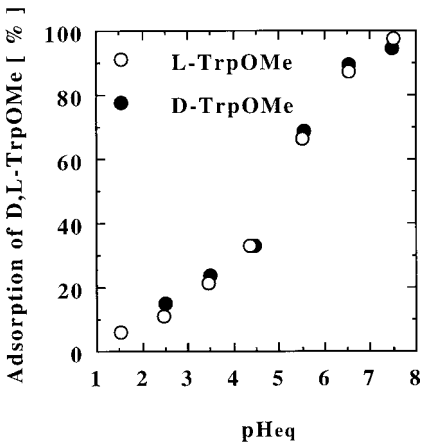

Figure 8 The pH dependence of the adsorption of D- and L-TrpOMe on the unimprinted polymer.

in the Trp. We deduced that the hydrogen bonding formation [NH (indole ring)—O═P] between the functional host molecule and the guest molecule is also an important element in achieving the enantioselectivity. In addition, the hydrophobic interaction between the methoxyl group and the polymer matrix is considered to enhance the enantiomeric selectivity. Of course these factors should be derived from the memorized recognition sites.

Binding Constants of Substrates on D- and L-TrpOMe Imprinted Polymers

We quantitatively characterized the template effect in the D and L imprinted polymers by evaluating the binding constants (K_α). The binding constants can be evaluated on the basis of the slope and intercept by the modified Scatchard plot. Table I lists the results of the binding constants for the TrpOMe imprinted polymers. The binding constant becomes an indicator to express an adsorption affinity of recognition sites for the target amino acid derivative. The D- and L-TrpOMe imprinted polymers exhibit high binding abilities to the corresponding imprinted guest molecules. To discuss the enantioselectivity quantitatively, we defined the separation factor as follows: $\alpha = K_{\alpha L(D)}/K_{\alpha D(L)}$. The separation factors on the D- and L-TrpOMe imprinted polymers were 1.4 and 1.5, respectively. These results indicate that the surface imprinting technique is very useful in creating enantioselective sites that can recognize the chirality of an optically active material on the polymer surface.

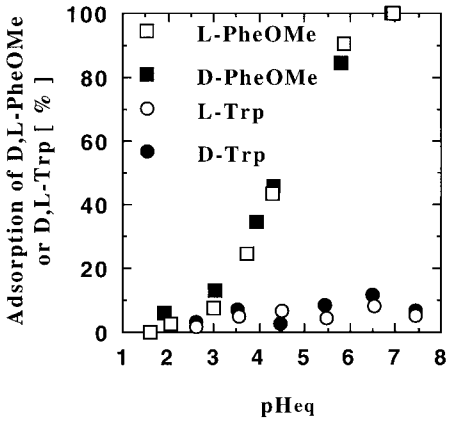

Figure 9 The pH dependence of the adsorption of D- and L-PheOMe or D- and L-Trp on the unimprinted polymer.

Table I Binding Constants of D- or L-TrpOMe on TrpOMe Imprinted Polymers

Imprinted Polymer and Substrate	Binding Constant $K(_\alpha$ (M^{-1})	Separation Factor α ($=K_{\alpha L(D)}/K_{\alpha D(L)}$)
L-TrpOMe		
L-TrpOMe	2.9×10^3	1.5
D-TrpOMe	2.0×10^3	
D-TrpOMe		
D-TrpOMe	4.8×10^3	1.4
L-TrpOMe	3.4×10^3	

Recognition Mechanism of Enantiomeric Selectivity

In a wide application of the imprinting technique it is important to elucidate the recognition mechanism of the imprinted polymer. We investigated the interactions between the target L-TrpOMe and the functional host molecule, *n*-DDP, at the prepolymerization stage by means of the FTIR and ^{1}H-NMR measurements. The characteristic peaks observed for the *n*-DDP–L-TrpOMe complex and L-TrpOMe were employed to infer the recognition morphology.

Table II shows the specific peaks on L-TrpOMe and its *n*-DDP complex. Based on the FTIR spectra, the N—H stretching vibration peak derived from the indole ring appeared at 3287 cm^{-1} on the L-TrpOMe and a similar peak on the *n*-DDP–L-TrpOMe complex had shifted to 3217 cm^{-1}. This result means that the hydrogen bonding interaction between P═O in the phosphonic part of *n*-

Table II FTIR Results by KBr Method

Band (cm^{-1})	Band Assignment
L-TrpOMe	
3287	N—H stretching vibration (indole ring)
1747	C═O stretching vibration
n-DDP–L-TrpOMe complex	
3217	N—H stretching vibration (indole ring)
1751	C═O stretching vibration

DDP and the proton (N—H) of the indole ring causes the lower shift when forming the complex. On the other hand, the sharp C═O stretching vibration peaks derived from L-TrpOMe and the *n*-DDP–L-TrpOMe complex appeared at 1747 and 1751 cm^{-1}, respectively. It can be presumed that the hydrogen bonding is not formed between the C═O group in the amino acid ester and the P—OH in the phosphonic group of the *n*-DDP.

Table III lists the results of ^{1}H-NMR on L-TrpOMe and the *n*-DDP–L-TrpOMe complex. In the case of forming the complex, we could not distinguish between methine and methylene proton peaks on the *n*-DDP–L-TrpOMe complex because of an overlapping of the methylene proton peak of *n*-DPP (—O—CH_2—). The indole proton (—NH) on L-TrpOMe was found at 11.45 ppm, and the peak on the complex had shifted to 11.28 ppm. This upfield chemical shift can be rationalized by the —NH—O═P interactions in forming the complex between *n*-DDP and L-TrpOMe. In addition, the resonance peak of the cationic amino group protons on the complex remarkably shifted toward the lower magnetic field (from 6.43 to 8.60 ppm) compared to that of the L-TrpOMe. It is known that the electrostatic interaction (—NH_3+—$^-$O—P) between *n*-DDP and L-TrpOMe causes a striking low-field chemical shift. The downfield and upfield chemical shifts are due to the shielding and antishielding effect of nearby phosphonic groups on the protons influenced by hydrogen bonding (—NH—O═P) and electrostatic (—NH_3^+—$^-$O—P) interactions.[36]

The enantioselective recognition morphology on the L-TrpOMe imprinted polymer is illustrated in Figure 10. There are four important factors for producing the enantioselectivity: the phosphonic group in the functional host molecule, *n*-DDP, is anchored on the surface of the imprinted polymer in such way that it can fit a desirable formation around L-TrpOMe when it interacts with the template; the electrostatic interaction between the functional host molecule and the substrate in a higher pH range; the hydrogen bonding interactions between the functional host molecule and the substrate; and the hydrophobic interaction between the methoxyl group in the substrate and the polymer matrix, DVB.

CONCLUSIONS

The D- and L-TrpOMe imprinted polymers containing an organophosphorus functional host mol-

Table III ^{1}H-NMR Results

δ (ppm)	Splitting	Assignment
L-TrpOMe		
11.45	s	—NH (indole ring)
7.0–8.0	m	—CH— (indole ring)
6.43	bs	—NH_3^+ (amino group)
4.39	t	—CH_2— (methylene group)
3.67	m	—CH— (methine group)
3.47	s	—OCH_3 (methoxyl group)
n-DDP–L-TrpOMe complex		
11.28	s	—NH (indole ring)
8.60	bs	—NH_3+ (amino group)
7.0–8.0	m	—CH— (indole ring or phenyl group)
3.35	s	—OCH_3 (methoxyl group)

The ^{1}H-NMR was performed at 250 MHz and 25°C with TMS as the internal standard and C_6D_6 (containing 15 vol % DMSO-d_6) as the solvent.

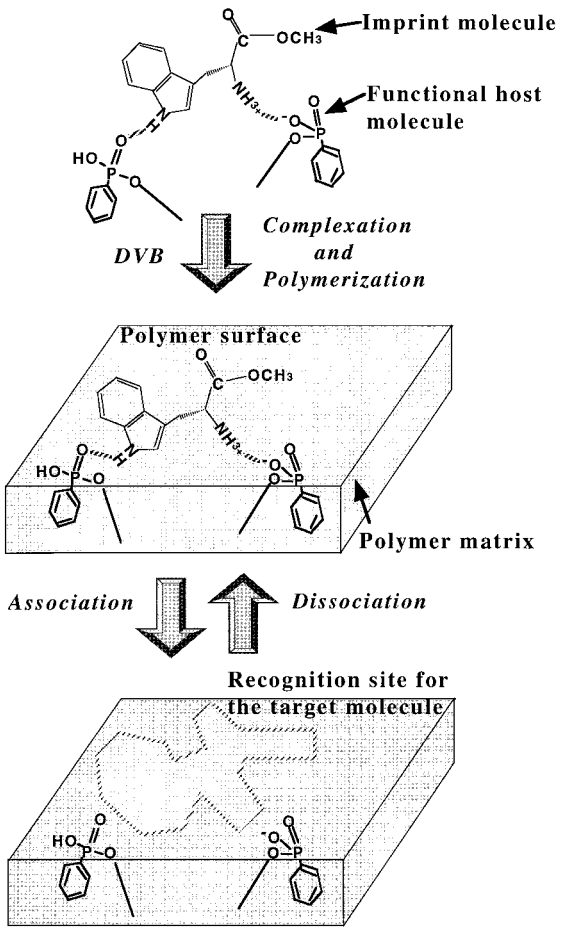

Figure 10 A schematic illustration of the surface of the L-TrpOMe imprinted polymer.

ecule showed a high enantioselectivity toward the corresponding imprint molecule. These enantioselectivities were supported by high binding constants of the complementary imprinted substrates that are suitable for the recognition sites on the surface of the imprinted polymers. In addition, the FTIR and ^{1}H-NMR studies provided useful information on the recognition mechanism by the template molecule. We are therefore concluding that, in order to have the driving forces in the enantioselectivity, the following three elements are required: an ionic interaction between the phosphonic part in the functional host molecule and the amino group in the substrate, hydrogen bonding formation between the indole ring and the functional P═O group, and a hydrophobic interaction between the methoxyl group in the amino acid ester and the polymer matrix. These factors can be enhanced by a rigid immobilization of the functional host molecules at a suitable location for a target guest molecule. We believe that the use of the surface template polymerization technique for the preparation of the "recognition polymers" will find various applications in the future.

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