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Molecular Imprinting of Cyclodextrin on Silica-Gel Support for the Stationary Phase of High-Performance-Liquid-Chromatography

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Abstract

By copolymerizing acryloyl cyclodextrin (CD) and N, N'-methylenebisacrylamide with vinylated silica-gel in the presence of a template in water, a thin layer of molecularly imprinted CD polymer was immobilized on a porous silica-gel support. This mechanically weak polymer was reinforced by the silica-gel and successfully used as a stationary phase in high-performance-liquid-chromatography (HPLC). When L-Phe-L-Phe was the template, the polymer/silica-gel conjugate retained L-Phe-L-Phe in the aqueous eluent more strongly than D-Phe-D-Phe. As expected, the D-Phe-D-Phe-imprinted polymer retained D-Phe-D-Phe more strongly than L-Phe-L-Phe. Selective separation of antibiotics was also achieved by the polymer/silica-gel conjugate. Molecularly imprinted CD polymer, immobilized on silica-gel, is an eminent stationary phase for HPLC in water.

Introduction

Recently, the molecular imprinting technique has been developed as a new methodology for synthesizing a tailor-made receptor for the target substrate [1]. As long as the target molecules are Ångstrom-size and the recognition is achieved in organic media, polymers of high activity and selectivity are successfully obtained and used as a stationary phase of HPLC [1]. The next theme for improving this technique is how to recognize nano-scaled large targets such as peptide, antibiotics, or protein efficiently in water as natural receptors do. The imprinting process must be carried out also in water. However, only limited examples are reported so far [2] because of the following barriers: (1) hydrogen bondings, which are preferentially used for the pre-organization of templates and functional monomers, are easily destroyed in water due to competition with the solvent [3], (2) the imprinted polymer is not stiff enough for the stationary phase of HPLC, since water-soluble conventional crosslinking agents such as N, N'-methylenebisacrylamide (MBAA) cannot sufficiently reinforce the polymer. For further development of molecular imprinting, these difficulties should be overcome.

In our previous works, the first problem has been solved by use of hydrophobic interactions instead of hydrogen bondings: we chose as functional monomers cyclodextrins (CDs) which bind Ångstrom-sized guests through apolar interaction in water [4]. Vinyl monomers of CDs were crosslinked with MBAA in the presence of large template molecules involving multiple recognition sites for CDs [5]. The obtained polymer efficiently recognized the template in water. The second problem (mechanistic strength of the polymer) has not yet been solved, although it is crucial for the application to a stationary phase of HPLC. Chemical modification of the crosslinking agent is not very successful: introduction of an aromatic moiety certainly makes the polymer stiff, but at the same time decreases the solubility in water [6].

In the present paper, we solve the second problem by preparing imprinted CD polymer on porous silica-gel. Stiffness is provided by the silica-gel support, and specific recognition is achieved by the thin layer of imprinted polymer. Silica-gel is an appropriate support here, since non-specific binding hardly occurs due to its intrinsic hydrophilic property. Therefore, the recognition behavior reflects only the binding property of the imprinted polymer. The imprinted polymer coated on the silica-gel strongly retains the template molecule and shows notable selectivity.

Experimental

Materials

6-O-α-D-Glucosyl-β-cyclodextrin (G1-β-CD) was purchased from Ensuiko Sugar Refining Co. (Japan). Its vinyl monomer (acryloyl-CD: Scheme 1) was synthesized by ester-exchange reaction with m-nitrophenyl ac-

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rylate according to the previous paper [5e, 7]. N, N'-Methylenebisacrylamide (MBAA), trichlorovinylsilane and other reagents were from Tokyo Kasei Co. The silica-gel used as a support was obtained from MACHEREY-NAGEL from Germany (Nucleosil 300-10: grain size 10 μm, pore size 30 nm in diameter, and specific surface area 100 $m^2\ g^{-1}$), and was dried at 140 °C for 1 day before use. Antibiotics, dipeptides, and amino acids (Figure 1) were obtained from Wako Pure Chemical Industries Co. (Japan) and BACHEM AG (Switzerland), and were used without further purification. Other reagents were purchased from Tokyo Kasei Co.

Introduction of vinyl groups on silica-gel surface

Vinyl groups were introduced on the surface of silica-gel according to the following procedure (see Scheme 1A): dried silica-gel (10 g) was dispersed into dry toluene-pyridine solution (110 mL, toluene/pyridine = 10/1 in volume) followed by dropwise addition of trichlorovinylsilane (250 μL, 2.0 mmol) under nitrogen. After the dispersion was stirred for 16 h at 50 °C, the silica-gel was collected and washed successively with chloroform, methanol, and water. Finally, the modified silica-gel was dried under vacuum and used for the immobilization of the polymer. The amount of vinyl group introduced into the silica-gel was quantified by the titration with $KMnO_4$, which revealed that 74 μmol of vinylsilane per 1 g was incorporated. This value corresponds to the reaction of about 10% of the total silanol groups on the surface of silica-gel. In order to avoid non-specific binding by undesirable hydrophobic interactions, surface coverage by vinylsilane was maintained at about 10%.

Immobilization of the imprinted polymer on the modified silica-gel

Molecular imprinting was carried out according to Scheme 1B. Acryloyl-CD (90 mg, 67 μmol), N, N'-methylenebisacrylamide (60 mg, 390 μmol) as a crosslinking agent, and the template molecule (30 μmol) was dissolved in 5 mM of tris(hydroxymethyl)aminomethane buffer solution (pH 8.0, 5 mL), and then vinylated silica-gel (600 mg) was dispersed. After stirring the dispersion for a few minutes, the polymerization was started by adding potassium persulfate (7 μmol, 2 mg) and N, N, N', N'-tetramethylethylenediamine (20 μmol, 3 μL) as an initiator-system under nitrogen at 37 °C for 1 h. Then the solid part was collected and washed with a large amount of water and subsequently with methanol to remove the template and unreacted monomers [8]. The conjugate of polymer and silica-gel obtained was directly packed into the column (*vide infra*).

As a control, non-imprinted CD polymer was immobilized on the silica-gel in the same manner described as above, except for the absence of the template. In order to elucidate the effect of CD, N, N'-methylenebisacrylamide was also immobilized on silica-gel in the absence of acryloyl-CD. HPLC analysis: The conjugate of polymer and silica-gel was packed in a stainless steel column tube (50 mm × 4.6 mm i.d, purchased from GL Science). The retention behavior of the substrate was monitored with a HPLC system (JASCO). As an eluent, 50 mM aqueous ammonium formate solution (pH 6.9) was used at a flow rate of 0.25 mL min^{-1}. Dipeptides (or amino acids) and antibiotics were detected by UV absorption at 258 and 277 nm, respectively.

Table 1. Retention times of various peptides and other guests by the Phe-Phe-imprinted CD polymers immobilized on the silica-gel support.

Guest	retention time/min		
	Non-Imp[a]	L-Phe-L-Phe-Imp	D-Phe-D-Phe-Imp
L-Phe-L-Phe	9.61	10.20	10.62
D-Phe-D-Phe	9.68	9.73	11.73
L-Phe	4.21	4.27	4.34
D-Phe	4.17	4.25	4.32
m-nitrophenol	24.65	21.47	23.65

[a]Polymer/silica-gel conjugate was prepared in the absence of template.

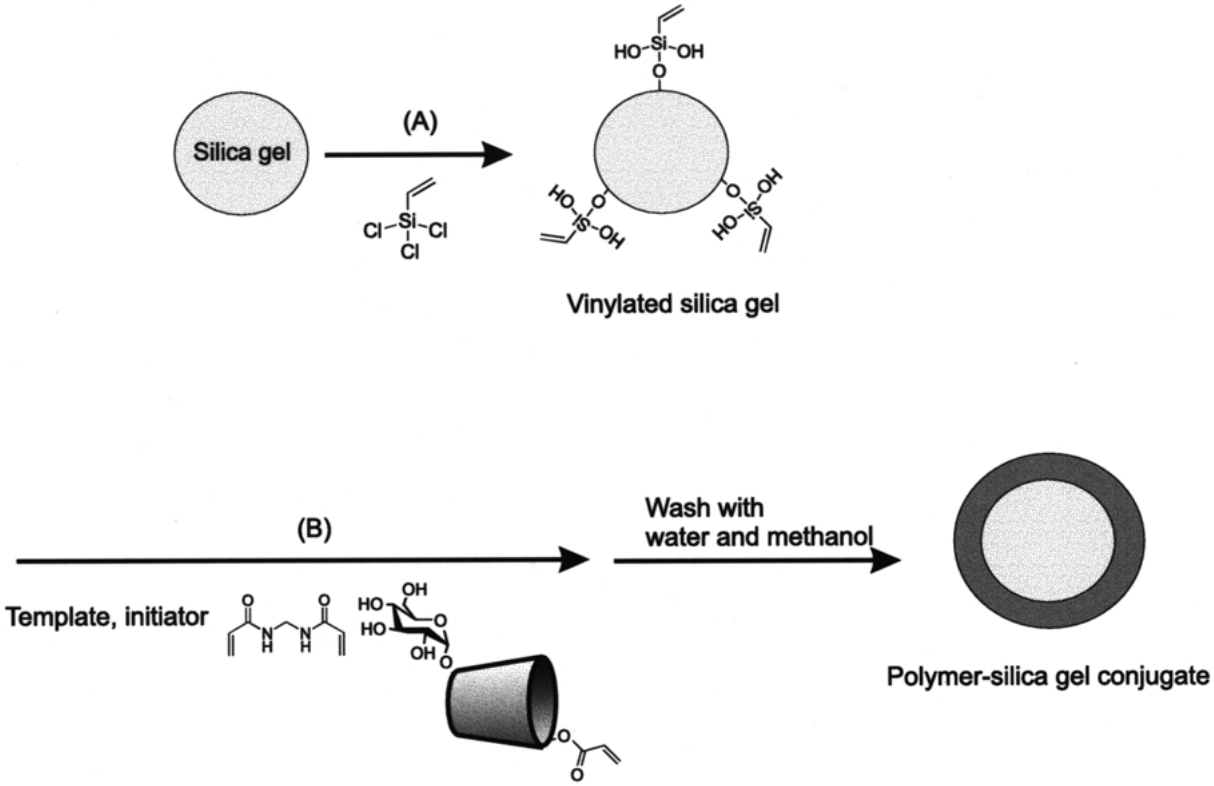
Results and discussion

Enantioselective recognition of dipeptides

In order to confirm the validity of the present methodology, enantiomers of Phe-Phe (D-Phe-D-Phe and L-Phe-L-Phe) were first used as templates (Table 1). Although CD itself is a chiral compound [9], the polymer/silica-gel conjugate prepared without template in this study (Non-imp) hardly discriminated these enantiomers. Exactly as designed, the molecular imprinting significantly affected the retention behavior. When L-Phe-L-Phe was used as a template, L-Phe-L-Phe was retained more strongly than D-Phe-D-Phe. With the conjugate prepared in the presence of D-Phe-D-Phe, D-Phe-D-Phe was retained much more strongly than its enantiomer. Thus, it could clearly be concluded that specific binding sites were successfully formed from CDs even in the molecular imprinting on the surface of silica-gel. Dipeptide structures are necessary for the specificity: D-Phe and L-Phe have almost the same retention times for both of the polymer/silica-gel conjugates. The possibility that only a half-part of the dipeptide was imprinted to the polymer is ruled out. These results support the imprinting mechanism in Scheme 2 which was proposed in the previous paper [5e]: multiple CDs are placed complementarily to the hydrophobic moieties of the template and the guest-binding is promoted by the cooperation of these interactions.

Role of the silica-gel support

Even without silica-gel, crosslinked polymer could be obtained by copolymerizing acryloyl-CD and MBAA as described in the Experimental section. When this polymer was directly packed into a stainless column and water was eluted to this column, however, the pressure of the pump soon exceeded its limit. These polymers are useless for the present purpose. With the polymer/silica-gel conjugate as the stationary phase of HPLC, however, the pump-pressure was



Scheme 1. Schematic procedure of the immobilization of the imprinted polymer on the silica-gel support. (A) Introduction of vinyl groups on the silica-gel surface. (B) Immobilization of the polymer on the vinylated silica-gel surface.

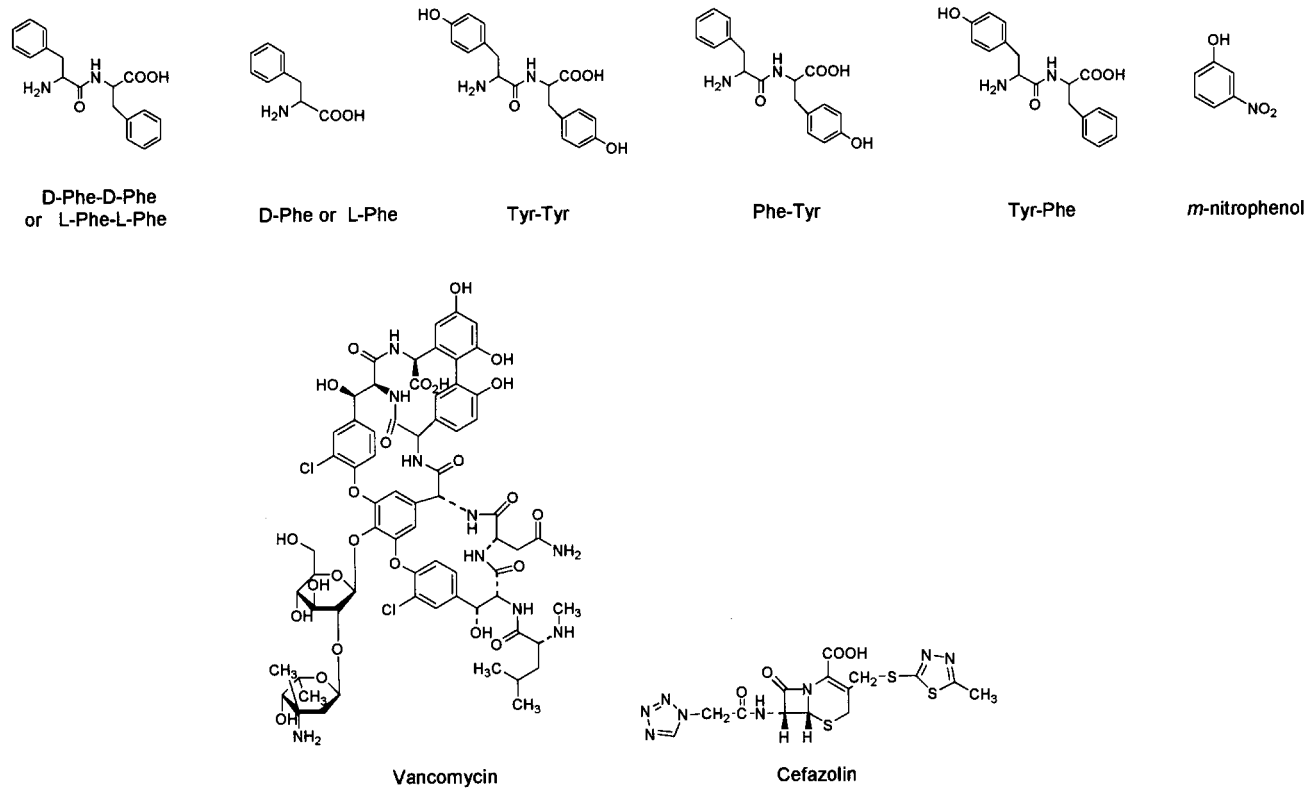
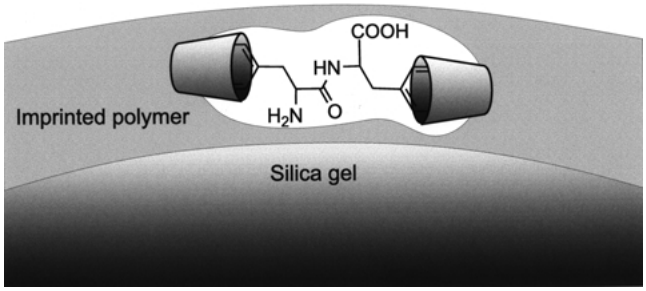


Figure 1. Template molecules and substrates used in the present study. L-Isomers were used for Phe-Tyr, Tyr-Phe, and Tyr-Tyr.



Scheme 2. Proposed structure of the binding sites formed on the silica-gel surface.

Table 2. Retention behaviour of various dipeptides and other guests by the polymer/silica-gel conjugate prepared from MBAA with or without acryloyl-CD.

Guest	Retention time/min[a]	
	MBAA[b]	MBAA + acryloyl CD[c]
L-Phe-L-Phe	4.24	9.61
D-Phe-D-Phe	4.25	9.68
Phe-Tyr	4.02	10.33
Tyr-Phe	4.06	10.22
Tyr-Tyr	4.04	10.92
L-Phe	3.88	4.21
D-Phe	3.89	4.17
m-nitrophenol	5.12	24.65

[a] A shock peak appeared at 2.4 min.
[b] *N*, *N*′-Methylenebisacrylamide was immobilized on the silica-gel surface without a template.
[c] Acryloyl-CD was copolymerised with MBAA on the silica-gel surface without a template.

always far below the limit and the HPLC was successfully achieved. The essential role of the silica-gel is evident.

The guest binding by the molecularly imprinted conjugate is ascribed to the CD residues. No retention was observed, when the silica-gel was coated with the homopolymer of MBAA (Table 2). Totally consistently, *m*-nitrophenol, which is known as an excellent guest for β-CD, was strongly retained by the conjugate prepared with acryloyl-CD. The layer of imprinted CD molecules, although it is really thin, shows the successful guest-separation [10].

Molecular imprinting towards antibiotics

This technique is also applicable to imprinting towards antibiotics (Table 3). By imprinting with vancomycin, the retention of this antibiotic is notably promoted, but the binding to another antibiotic cefazolin is hardly affected. The difference in the retention times between these two antibiotics is about 5 min. With cefazolin as the template, however, the retention towards cefazolin is selectively promoted, and a cefazolin-selective column is now obtained. Without the imprinting, the polymer/silica-gel conjugate retains both of the antibiotics with comparable strength. These results indicate that a variety of water-soluble biomolecules are available as templates for the present molecular imprinting.

In conclusion, a thin layer of molecularly imprinted polymers of cyclodextrins has been successfully immobilized on the silica-gel surface in water. The polymer/silica-gel conjugates obtained efficiently recognize the template molecules as a stationary phase of HPLC. This technique should be also applicable to other functional monomers.

Table 3. Retention time of antibiotics by the imprinted CyD polymer immobilized on the silica-gel support.

Substrate	Retention time/min		
	Non-Imp[a]	Van-Imp[b]	Cef-Imp[b]
Vancomycin	10.32	14.66	9.87
Cefazolin	9.32	9.73	11.09

[a] Polymer/silica-gel conjugate was prepared in the absence of template.
[b] The templates are vancomycin and cefazolin, respectively.

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6. Non-specific binding is enhanced when an aromatic moiety is introduced into the crosslinking agent. Note that conventional imprinting requires much excess crosslinking agent compared with the functional monomer.
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8. Even if all the monomers fed into the reaction mixture are immobilized on the silica-gel, the surface occupation should be 0.25 nm^2 per one MBAA molecule and 1.5 nm^2 per one acryloyl-CD molecule. Thus, the polymer on the surface of the silica-gel should be a unimolecular layer or bilayer.
9. Intrinsically, β-CD prefers D-Phe-D-Phe to its enantiomer: see Ref. 5e.
10. All the substrates used here have aromatic groups which form complexes with CD in water.