Journal of Inclusion Phenomena and Macrocyclic Chemistry (2006) 56: 39–44
DOI 10.1007/s10847-006-9058-5

Improved Method of Molecular Imprinting of Cyclodextrin on Silica-gel Surface for the Preparation of Stable Stationary HPLC Phase

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Key words: amino acid, cyclodextrin, immobilization, HPLC, molecular imprinting, stationary phase

Abstract

By using *N*-(3-triethoxysilyl)propylacrylamide (TPAAm), vinyl groups were introduced onto the surface of silica-gel. On the surface of this silica-gel, β-CyD was molecularly imprinted by using a redox initiator, and the composite was used as stationary phase of high performance liquid chromatography (HPLC). The pump pressure was sufficiently low and did not increase even after continuous elution for 24 h. In order to prepare still more stable columns, a new polymerization process was developed. There, the redox initiator was first mixed with the surface-modified silica-gel and then vinylated β-CyD, crosslinker, and the template were added. This modification promoted the immobilization of β-CyD copolymer to the silica-gel, resulting in still lower pump pressure. Concurrently, the imprinting efficiency was increased in comparison with previous method where the redox initiator was directly added to the mixture of the β-CyD–template complex, crosslinker, and surface-modified silica-gel. The molecularly imprinted β-CyD column, prepared by this new method, efficiently discriminated the enantiomers of *N*-benzyloxycarbonyltyrosine.

Introduction

Molecular imprinting is one of the most promising methodologies for the preparation of tailor-made artificial receptors that can selectively bind target molecules [1]. This method is based on the polymerization of functional monomers in the presence of a template. After removing the template, the ''cavity'' left in the polymer selectively binds the target molecule (= template) because the functional monomers in the cavity should be located complementarily to the template. Because the concept of molecular imprinting is comprehensive and the method is easy, various molecularly imprinted polymers (MIPs) have been reported. Most of these MIPs were synthesized in aprotic organic solvents such as chloroform in which functional monomers were pre-organized through hydrogen bondings. Ethylene glycol dimethacrylate or divinylbenzene was mostly used as a crosslinker. The MIPs prepared with these crosslinkers are sufficiently stiff to be used as the stationary phase of HPLC. In contrast, preparation of MIPs in water has been rather scarce, because (1) pre-organization of functional molecules through hydrogen bondings is very difficult in water, and (2) conventional aqueous crosslinker such as *N*,*N*′-methylenebisacrylamide (MBAAm) does not provide enough stiffness to the MIP [2]. For the imprinting in water, at least these two difficulties should be overcome.

Previously [3], we prepared MIPs of cyclodextrin (CyD) in bulk water. CyD was first vinylated and copolymerized with MBAAm as a crosslinker. In batchwise experiments, the MIPs efficiently and selectively bound the template (dipeptides or antibiotics). Although the imprinted CyD polymers themselves were rather soft and hardly available to a stationary phase of HPLC, we solved this problem by achieving the molecular imprinting on the surface of silica-gel support. There, silica-gel was first vinylated by trichlorovinylsilane (TCVS), and treated with both vinylated CyD and MBAAm as shown in Scheme 1. The packed column efficiently discriminated the template from other guests [4]. Thus, both imprinting in water and sufficient stiffness for stationary phase of HPLC were simultaneously fulfilled by this method. Other advantages of this method are as follows. First, amounts of the monomers and templates required for the imprinting are

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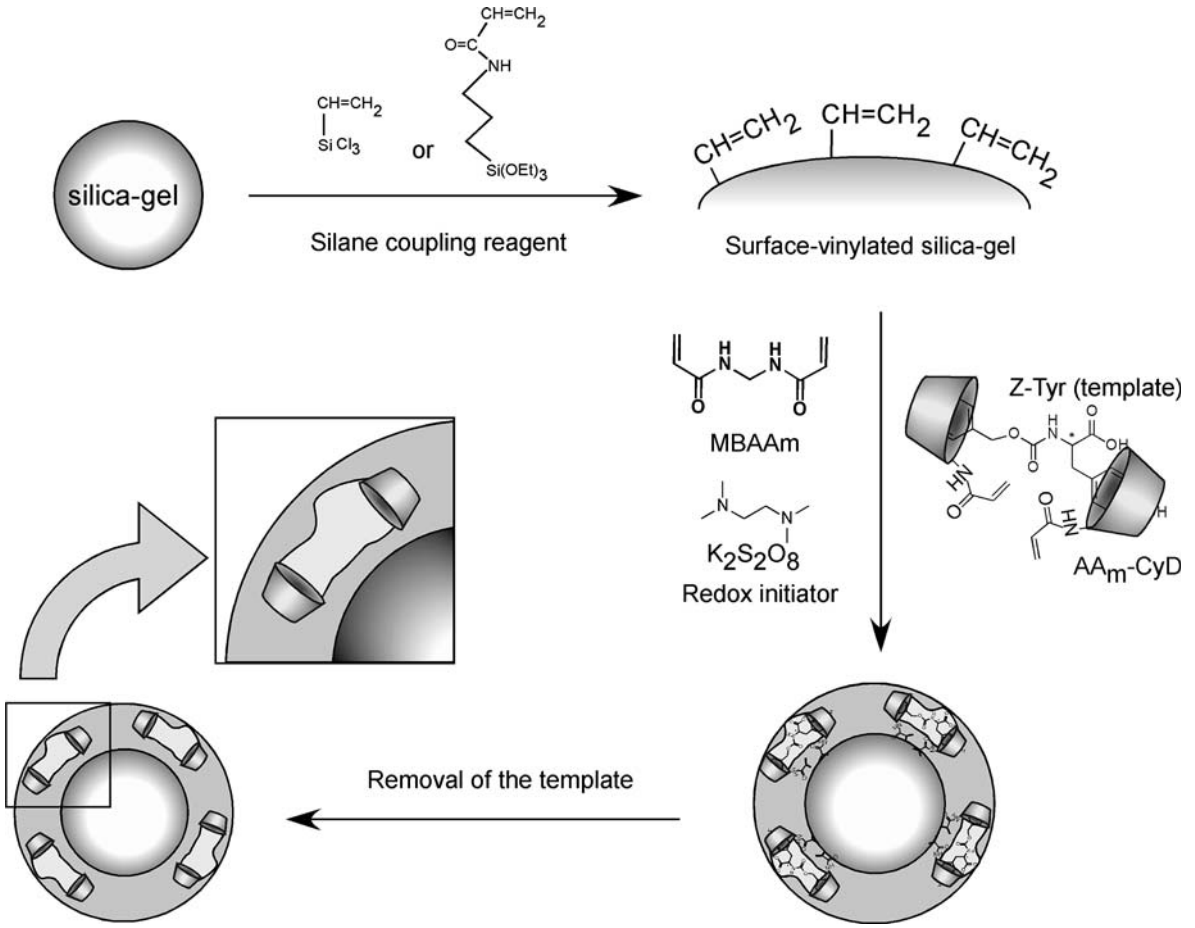


Scheme 1. Procedure of the immobilization of imprinted β-CyD polymer on silica-gel.

much less than those in conventional imprinting, and therefore, expensive templates or functional monomers are available. Second, HPLC peaks are relatively sharp since silica-gel particles of uniform size are commercially available. Thus, surface modification of silica-gel is one of the effective techniques for the immobilization of imprinted polymers.

However, trichlorovinylsilane (TCVS) has several problems as a surface modifier. One of them is its low polymerization reactivity. Accordingly, some of the imprinted CyD polymer was not covalently immobilized on the silica-gel and thus gradually removed from the stationary phase. As a result, the pump pressure concurrently increased. The second problem is its high hydrophobicity. Modification with TCVS made the silica-gel surface so hydrophobic that water was repelled from the modified particles. Therefore, we could not introduce sufficient amounts of vinyl groups onto the surface without losing the miscibility with water. In the present paper, *N*-(3-triethoxysilyl)propylacrylamide (TPAAm) involving more reactive acryloyl group (depicted in Scheme 1) is synthesized as a surface modifier and is applied to the vinylation of silica-gel. On the surface of these vinylated silica-gel particles, β-cyclodextrin (β-CyD) is imprinted to the enantiomers of *N*-benzyloxycarbonyltyrosine (*Z*-L-Tyr and *Z*-D-Tyr). Highly stable HPLC columns of imprinted β-CyD polymer are prepared. Furthermore, a new polymerization process, in which the vinylated silica-gel is first treated with redox initiator in the absence of CyD–template complex and crosslinker, is developed. Interestingly, both column stability and imprinting efficiency are considerably higher than those in conventional method (direct reaction of the monomer mixture with the redox initiator).

Experimental

Materials

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. *Z*-L-Tyr and *Z*-D-Tyr were obtained from Wako Pure Chemical Industries Co. (Japan), and were used without further purification. β-CyD, MBAAm, and other reagents were from Tokyo Kasei Co.

Synthesis of N-(3-triethoxysilyl)propylacrylamide (TPAAm)

To a chloroform solution (300 mL) of 3-aminopropyltriethoxysilane (10 g: 45 mmol) and triethylamine

(13 mL: 93 mmol) on ice bath, acryloyl chloride (4.1 g: 45 mmol) was added dropwise. After stirring for 3 h at room temperature, the solvent was evaporated and the crude oil was subjected to silica-gel chromatography (eluent: chloroform/methanol = 9/1). Yield: 53% (6.6 g). ^{1}H-NMR [$CDCl_3$, 500 MHz] δ = 8.07 (bs, 1H, –CO–N*H*–), 6.21 (dd, 1H, ^3J(H,H) = 17.2 Hz, ^3J(H,H) = 10.1 Hz, CH_2 = C*H*–CO–), 6.07 (dd, 1H, ^3J(H,H) = 17.2 Hz, ^2J(H,H) = 2.3 Hz, CH_2 = CH–), 5.56 (dd, 1H, ^3J(H,H) = 10.1 Hz, ^2J(H,H) = 2.3 Hz, CH_2 = CH–), 3.75 (q, 6H, ^3J(H,H) = 7.0 Hz, –OCH_2CH$_3$), 3.10 (m, 2H, –NH–CH_2–), 1.48 (m, 2H, –NH–CH$_2$–CH_2–CH$_2$–), 1.15 (t, 9H, ^3J(H,H) = 7.0 Hz, –OCH$_2$CH_3), 0.55 (t, 2H, ^3J(H,H) = 8.5 Hz, –NH–CH$_2$–CH$_2$–CH_2–).

Synthesis of mono-3-(N-acrylamido)-3-deoxy-altro-β-cyclodextrin (AAm-CyD)

The β-CyD monomer AAm-CyD was synthesized as follows, through tosyl-β-CyD and mono-3-amino-3-deoxy-altro-β-CyD [5]. First, β-CyD (100 g) dried under vacuum at 45 °C was dissolved in dry dimethylformamide (DMF, 500 mL). Then sodium hydride (3.5 g) was added and resulting mixture was stirred overnight, followed by dropwise addition of DMF solution of tosyl chloride (16.8 g). After 30 min of stirring at room temperature, water was added to terminate the reaction. Then the reaction mixture was poured into acetone, and white precipitates were collected. Obtained precipitates were dissolved in water and mono-substituted tosyl-β-CyD was separated by polystyrene column chromatography (HP-20) with 20–25% aqueous methanol solution as eluent. Yield: 36% (41 g).

Tosyl-β-CyD thus obtained (16.8 g) was dissolved in aqueous NH_3 (28%) solution (300 mL) and the reaction mixture was kept for 24 h at room temperature. Then the solvent was evaporated and resulting crude solid was purified by cation exchange resin (DIAION PK208) with 3% aqueous NH_3 solution as eluent to obtain mono-3-amino-3-deoxy-altro-β-CyD. Yield: 58% (8.5 g).

DMF solution (10.6 mL) of *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphonate (HBTU, 1.8 g), acrylic acid (0.33 mL), and *N,N*-diisopropylethylamine (1.6 mL) was added to the mono-3-amino-3-deoxy-β-CyD (5.3 g) in DMF (53 mL), and the resulting mixture was stirred for 24 h at room temperature. Then the solvent was evaporated and crude oily product was dissolved in a small amount of water, and then poured into acetone for the precipitation. The white solid was recollected and was further subjected to column chromatography on Sephadex G-15 (5 × 200 cm) to purify AAm-CyD. Yield: 29% (1.6 g): MALDI-TOFMS (positive mode): obsd. 1211.0 (calcd. for [M + Na$^+$]: 1210.4). ^{1}H-NMR [D_2O, 500 MHz] δ = 6.20 (m, 2H), 5.73 (d, 2H, J = 11 Hz), 5.1–4.9 (m, 7H), 4.25 (m, 1H), 4.15 (bs, 1H), 4.0–3.5 (m, 40H).

Introduction of vinyl groups on silica-gel surface

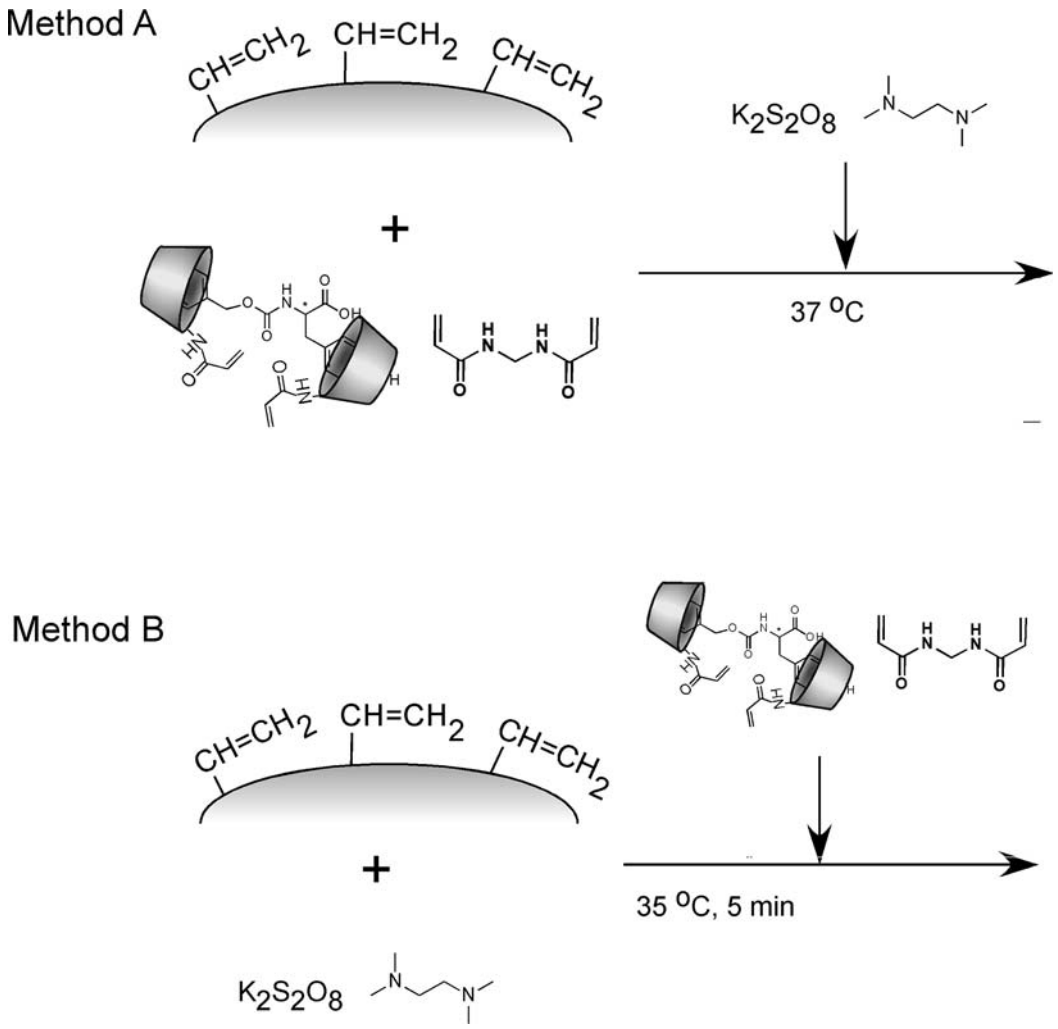
Use of N-(3-triethoxysilyl)propylacrylamide (TPAAm)
Dried silica-gel (10 g) was dispersed in dry xylene (200 mL) and 568 μL (0.2 mmol) of TPAAm was added to this dispersion, followed by vigorous stirring for 24 h at 50 °C. Then the silica-gel was collected and washed successively with methanol, water, and methanol again. Finally, the modified silica-gel was dried under vacuum and used to immobilize the polymer. According to the titration with $KMnO_4$, about 90 μmol of vinyl groups was incorporated to 1 g of the silica-gel. It should be noted that silica-gel thus obtained had sufficient miscibility with water.

Use of trichlorovinylsilane (TCVS)
Dried silica-gel (10 g) was dispersed in dry toluene–pyridine solution (110 mL, toluene/pyridine = 10/1 in volume), followed by dropwise addition of TCVS (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. About 70 μmol of TCVS was incorporated per 1 g of the silica-gel. Further incorporation of TCVS made the surface so hydrophobic that water was repelled.

Immobilization of the imprinted β-CyD polymer on the surface of modified silica-gel (Scheme 2)

Method A (previous method)
In 50 mM of tris(hydroxymethyl)aminomethane (Tris) buffer solution (pH 8.0, 5 mL), AAm-CyD (90 mg, 67 μmol), MBAAm (60 mg, 390 μmol) as a crosslinking agent, and template molecule (30 μmol) were dissolved, and then the vinylated silica-gel prepared above (600 mg) was dispersed in this solution. After stirring the dispersion for a few minutes, the polymerization was started under nitrogen at 37 °C by adding the mixture of potassium persulfate (7 μmol, 3 mg) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED: 20 μmol, 3 μL) as an initiator-system. After 1 h, the solid part was collected and washed with large amount of water and subsequently with methanol to remove the template and unreacted monomers. Thus obtained silica-gel/polymer composite was directly packed into the column. In the present study, enantiomers of *N*-benzyloxycarbonyltyrosine (*Z*-L-Tyr or *Z*-D-Tyr, see Scheme 1) was used as templates (and also as guests).

Method B (new method)
Vinylated silica-gel (600 mg) was dispersed in degassed Tris buffer solution (2 mL) under nitrogen atmosphere, and potassium persulfate (300 μmol, 100 mg) and TEMED (200 μmol, 30 μL) were successively added. Larger amount of redox initiator was employed to accelerate the polymerization. The dispersion was



Scheme 2. Two polymerization methods for immobilization of imprinted β-CyD polymer on the silica-gel. Method A: the vinylated silica-gel, monomers, and template are mixed in the buffer solution and the redox initiator is directly added to the mixture. Method B: the vinylated silica-gel is first treated with the redox initiator and then a buffer solution of monomers and template is added to the mixture.

degassed again and kept at 35 °C for 5 min under nitrogen to react the redox initiator with the vinyl groups on the silica-gel. Then AAm-CyD (90 mg, 67 μmol), MBAAm (60 mg, 390 μmol), and template molecule (30 μmol) were added and the dispersion system was stirred under nitrogen at 35 °C for 24 h. The solid part was collected and washed with large amount of water and subsequently with methanol to remove the template and unreacted monomers. The product was directly packed into the column.

As a control, non-imprinted CyD polymers were immobilized on the modified silica-gel in the same manner described above, except for the absence of the template.

HPLC analysis

The silica-gel, coated with the imprinted β-CyD polymer, was packed in a stainless steel column tube (50 mm × 4.6 mm i.d., purchased from GL Science). Retention behavior of guests was monitored with an HPLC system (JASCO). As an eluent, 50 mM Tris buffer solution (pH 8.0) was used at flow rate of 0.25 or 0.50 mL min^{-1}. Twenty microliters of 1 mM aqueous guest solution (*Z*-L-Tyr or *Z*-D-Tyr) was injected to the HPLC, and eluted guest was detected by UV absorption at 260 nm. The capacity factor k was calculated according to the following equation:

$$k = (t_1 - t_0)/t_0 \quad (1)$$

where t_1 and t_0 are the retention times of substrate and acetone (as void marker), respectively. Enantioselectivity α was estimated from the capacity factors of *Z*-D-Tyr and *Z*-L-Tyr.

Evaluation of the immobilization efficiency and confirmation of the removal of template from the imprinted β-CyD polymer

After the polymerization, both supernatant of the dispersion and the solvent used to wash the modified silica-gel were collected into the flask and evaporated. To this dried flask, was added desired amount of water, and the resulting solution was analyzed by reversed-phase

HPLC (Merck LiChrospher 100 RP-18(e) column) with water/acetonitrile = 7/3 containing 1% trifluoroacetic acid as mobile phase. By monitoring the absorbance at 260 nm, the amounts of unreacted monomers and removed template were quantified.

Results and discussion

New silane coupling reagent for efficient immobilization of imprinted β-CyD polymer to silica-gel

First, the surface of silica-gel was modified by either TCVS or TPAAm, and the efficiency of immobilization of imprinted β-CyD polymer to the silica-gel was investigated. Method A was employed. Here, all the components of the imprinted β-CyD polymer (AAm-CyD, the vinylated silica-gel, MBAAm) were dispersed in the buffer solution under nitrogen, and then the redox initiator (potassium persulfate/TEMED system) was added to start the polymerization (see Scheme 2). Under these conditions, most (>95%) of AAm-CyD and MBAAm were consumed for the polymerization [6].

In the initial stage of the HPLC experiments, even the columns, prepared with TCVS-coated silica-gel, were successfully used as stationary phase; the initial pressure was rather low (200 kg/cm^2, flow rate; 0.25 mL/min, see Table 1) and kept sufficiently lower than the limit for several hours. But the pressure gradually increased and exceeded the limit after 24–30 h. This fact indicates that some portion of the AAm-CyD/MBAAm copolymer, bound to the silica-gel, was not immobilized to the silica-gel by covalent linkages, and rather was just non-covalently adsorbed onto the surface of silica-gel. This non-covalently bound polymer was gradually removed from the silica-gel to block the flow. Consistently, when unmodified silica-gel (having no vinyl groups) was used in place of the vinylated silica-gel and the copolymer was never covalently immobilized on the surface of silica-gel, the pump pressure soon (in a few hours) exceeded the limit.

The insufficient immobilization described above is primarily attributed to the low reactivity of TCVS in which a vinyl group is directly bound to silica-gel. The polymerization is suppressed by steric hindrance. Thus, TPAAm having still more reactive acryloyl group was synthesized and used as a surface modifier. Exactly as designed, the pressure of pump was much lower for TPAAm (the initial value = 85 kg/cm^2), and scarcely increased upon continuous flow of the eluent. Apparently, the copolymer was more efficiently immobilized on the surface of the silica-gel through covalent linkages. Under non-imprinting conditions, the capacity factors k for TCVS- and TPAAm-modified silica-gels were similar as shown in Table 1.

Table 1. Effect of silane coupling reagent on the HPLC pressure for the columns of β-CyD polymer-coated silica-gel[a]

Reagent	Capacity factor for *Z*-L-Tyr[b]	Initial pressure (kg cm^{-2})	Immobilization process
None	5.21	170[c]	Method A
TCVS	4.35	200[d]	Method A
TPAAm	4.46	85	Method A
TPAAm	3.02	34	Method B

[a]Flow rate of the elution was 0.25 mL min^{-1}.
[b]Stationary phase was prepared in the absence of template molecule.
[c]The pressure exceeded the limit (300 kg cm^{-2}) after several hours of elution.
[d]After 24 h, the pressure exceeded the limit.

Improved method for the molecular imprinting (prior treatment of vinylated silica-gel with the redox initiator: Method B)

In Method A, the redox initiator was directly added to the dispersion of the monomers and modified silica-gel. Therefore, the initiation radicals are primarily formed in the solution. If the polymerization is terminated before the growing radical reacts with the vinyl groups on the surface of the silica-gel, the copolymer is not covalently immobilized to the silica-gel. These polymers are bound to the silica-gel simply by non-covalent interactions. When this modified silica-gel is used for HPLC, these polymers are gradually removed from the silica-gel and induce the increase of pump pressure.

Ideally, the polymerization should start on the surface of the silica-gel and proceed only there. In order to realize this situation, a new polymerization method (Method B) was developed. Here, the redox initiator was first added to aqueous dispersion of the TPAAm-modified silica-gel (in the absence of the monomers) and the resulting dispersion was kept for 5 min to transfer the radicals to the acryloyl groups on the silica-gel (see Scheme 2). Then the monomers (AAm-CyD, MBAAm, and the template) were added to this dispersion for the copolymerization to proceed. The polymerization successfully proceeded, and the resulting copolymer-silica gel composite was applied to HPLC. Interestingly, the pump pressure was greatly lowered from 85 kg/m^2 in Method A to 34 kg/m^2 (see bottom of the Table 1) [7].

Effect of the polymerization method on the imprinting to amino acid

New polymerization method (Method B) was applied to the imprinting of *Z*-Tyr for the separation of its enantiomers. Since the pressure of the column prepared by Method B was sufficiently low, elution rate could be raised from 0.25 to 0.50 mL/min. Without template molecule, capacity factors of *Z*-L-Tyr and *Z*-D-Tyr were almost the same (Table 2). The enantioselectivity α, defined as the ratio of the retention time of *Z*-D-Tyr with respect to that of *Z*-L-Tyr, was nearly 1.0 (the HPLC charts are presented in Figure 1a). Slightly larger retention time towards the D-form is attributed to the chirality of β-CyD.

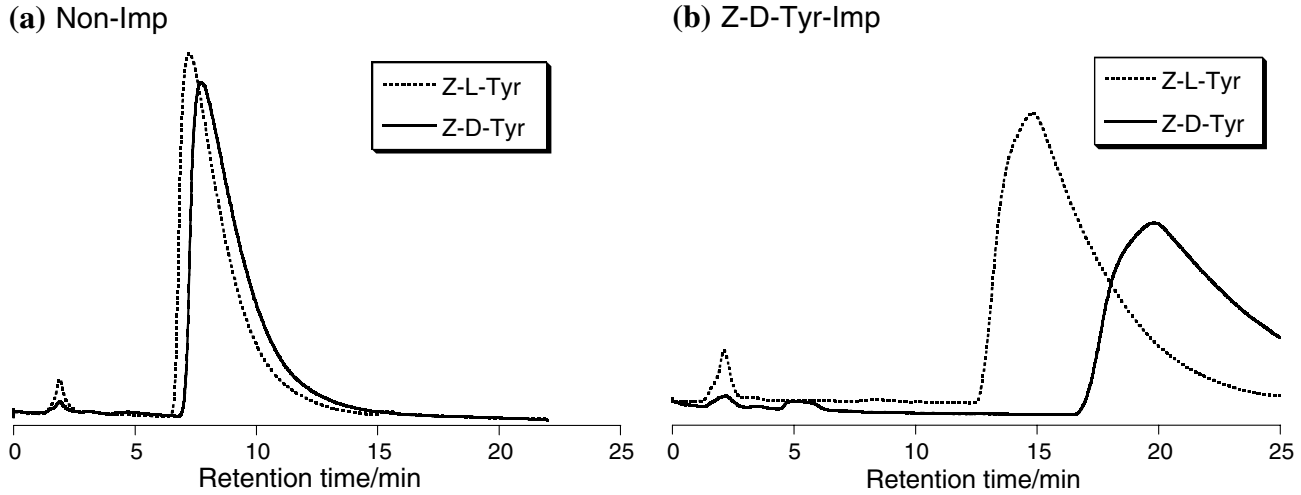


Figure 1. Retention behaviors of the enantiomers of *Z*-Tyr with (a) non-imprinted and (b) *Z*-D-Tyr-imprinted *β*-CyD column prepared by Method B (silica modifier = TPAAm). Tris buffer solution (pH 8.0; 50 mM) was eluted to the column at flow rate of 0.5 mL/min.

Table 2. Enantioselective recognition of *Z*-Tyr with imprinted *β*-CyD polymer prepared by Method B[a]

Substrate	Capacity factor *k*		
	Non-Imp	L-Imp[b]	D-Imp[c]
Z-L-Tyr	3.02	7.08	7.12
Z-D-Tyr	3.29	6.65	9.85
Enantioselectivity α[d]	1.09	0.94	1.38

[a]Flow rate was 0.5 mL min^{-1}.
[b]*Z*-L-Tyr was used as a template.
[c]*Z*-D-Tyr was used as a template.
[d]Ratio of the retention time of *Z*-D-Tyr with respect to that of *Z*-L-Tyr.

When *Z*-D-Tyr was used as the template for molecular imprinting, however, the column retained *Z*-D-Tyr much more strongly than *Z*-L-Tyr: the capacity factors of *Z*-D-Tyr and *Z*-L-Tyr were 9.85 and 7.12, respectively (see also Figure 1b). The enantioselectivity α was 1.38. As expected, the imprinted *β*-CyD polymer prepared in the presence of *Z*-L-Tyr bound this enantiomer more strongly than *Z*-D-Tyr, and thus α was reversed from 1.34 to 0.94. It should be noted that Method B also increased the molecular imprinting efficiency compared with our previous Method A. In Method A, imprinting of *Z*-D-Tyr could raise the retention time of *Z*-D-Tyr, but its imprinting efficiency was rather small. As a result, α of the *Z*-D-Tyr imprinted polymer, prepared by Method A, was only 1.12, which was much lower that accomplished by using Method B.

In conclusion, *N*-(3-triethoxysilyl)propylacrylamide (TPAAm), which has a highly reactive acryl group, was applied to the vinylation of silica-gel. When the silica-gel was treated with this surface modifier and imprinted *β*-CyD polymer was immobilized on this surface, the pump pressure in HPLC was sufficiently low. Furthermore, new polymerization process (treatment of the surface-modified silica-gel with the redox initiator before the addition of monomers) was developed and both stability of the column (low pump pressure) and high imprinting efficiency were simultaneously fulfilled.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The support by Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency (JST) (for H.A) is also acknowledged.

References

1. M. Komiyama, T. Takeuchi, T. Mukawa, and H. Asanuma: *Molecular Imprinting*, Wiley-VCH, Germany (2003).
2. (a) L.I. Andersson: *Anal. Chem.* **68**, 111 (1996); (b) A. Kugimiya, T. Takeuchi, J. Matsui, K. Ikebukuro, K. Yano, and I. Karube: *Anal. Lett.* **29**, 1099 (1996).
3. (a) H. Asanuma, K. Kajiya, T. Hishiya, and M. Komiyama: *Chem. Lett.* **665** (1999); (b) H. Asanuma, T. Akiyama, K. Kajiya, T. Hishiya, and M. Komiyama: *Anal. Chim. Acta.* **435**, 25 (2001).
4. T. Akiyama, T. Hishiya, H. Asanuma, and M. Komiyama: *J. Inclusion Phenom. Macrocyclic Chem.* **41**, 149 (2001).
5. (a) A.R. Khan, P. Forgo, K.J. Stine, and V.T. D'Souza: *Chem. Rev.* **98**, 1977 (1998); (b) D. Rong and V.T. D'Souza: *Tetrahedron Lett.* **31**, 4275 (1990); (c) T. Murakami, K. Harata, and S. Morimoto: *Chem. Lett.* **553** (1988).
6. In the presence of *Z*-D-Tyr or *Z*-L-Tyr, however, about 20% of the initially charged AAm-CyD was detected in the supernatant. It is probably attributed to the inhibition of polymerization by steric hindrance of the template which is complexing with AAm-CyD. Similarly, 20% of unreacted AAm-CyD was also detected in Method B when *Z*-Tyr was used as the template for the molecular imprinting.
7. The amount of immobilized AAm-CyD was not much changed between Method A and Method B, as judged from the analysis of unreacted AAm-CyD in the supernatant.