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β-Cyclodextrin (β-CyD)-based polymeric receptors for γ-endorphin (γ-endor, an opioid heptadecapeptide) were prepared using the molecular imprinting method. When mono-3-(*N*-acrylamido)-3-deoxy-β-CyD bearing a vinyl group in the secondary hydroxyl side of the cavity of β-CyD was polymerised in water in the presence of γ-endor, the binding activity of the β-CyD polymer to this peptide in water was enormously promoted by the imprinting. By contrast, the bindings towards methionine–enkephalin (N-terminal pentapeptide of γ-endor) and its homologue leucine–enkephalin were suppressed. Thus, the binding of γ-endor by the imprinted polymer was highly selective. The imprinting towards γ-endor was also successful with the use of the β-CyD monomer bearing a vinyl group in the primary hydroxyl side of the cavity, although the recognition was less strict. Various factors affecting the imprinting efficiency (kinds of β-CyD vinyl monomer and template, as well as the pH of imprinting mixture) are discussed.

Keywords: cyclodextrins; molecular imprinting; oligopeptides; column

1. Introduction

Preparation of artificial receptors that selectively bind target biomacromolecules (e.g. proteins, nucleic acids and others) has been attracting interest of chemists and biochemists because of their potential applications to separation, purification, diagnosis and many others. However, a general strategy for the preparation of these receptors has not yet been available. To date, a number of artificial receptors for small guests have been elegantly designed and synthesised (*1–7*). On the other hand, receptors for large guest molecules are still very difficult to prepare, since several functional groups must be placed precisely at distant positions. Furthermore, they must work in bulk water, imposing additional restraints in their molecular design. Accordingly, there still remains a considerable gap between chemically obtained molecular recognition systems and naturally occurring ones.

Molecular imprinting is one of the most widely employed techniques for preparing molecular receptors (*8–14*). By polymerising functional monomers in the presence of a template molecule, various polymeric receptors can be prepared in a tailor-made fashion. Many elegant imprinting systems using proteins as templates were reported (*15–20*). However, few successes have been made in the preparation of receptors which recognise proteins in water. In order to achieve both imprinting of biomacromolecules and their binding in bulk water, we recently proposed to use cyclodextrin (CyD) as a functional monomer which binds various guests in water (*21–30*). In the imprinting processes, several CyD molecules are placed at predetermined positions in a rigidly defined polymeric structure so that they cooperatively bind the target guest. By using this CyD imprinting method, receptors for amino acids, oligopeptides, antibiotics and steroids were successfully prepared (*25–31*). However, oligopeptides used previously as templates and guests have been mostly limited to rather small ones (the number of amino acids < 4) (*32*, *33*), and the information on the imprinting towards larger oligopeptides in water has been still insufficient.

In this paper, β-CyD-based polymeric receptors for physiologically active oligopeptides are prepared using the molecular imprinting method. Three oligopeptides having neuroleptic-like activity are used as the templates (Figure 1). γ-Endorphin (γ-endor) is a well-known endogenous opioid heptadecapeptide (*34*). On the other hand, methionine–enkephalin (Met–Enk) is a pentapeptide that has the same sequence as the N-terminal five amino acid residues of γ-endor. The $Met^5 \rightarrow Leu^5$ homologue of Met–Enk is leucine–enkephalin (Leu–Enk). As the functional monomers, two kinds of vinyl monomers of β-CyD, mono-3-(*N*-acrylamido)-3-deoxy-β-CyD (3-AAm-CyD) and mono-6-(*N*-acrylamido)-6-deoxy-β-CyD (6-AAm-CyD), are used to examine the effect of the position of the vinyl group on the imprinting efficiency (their structures are also

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γ-endorphin Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu
Met-enkephalin Tyr-Gly-Gly-Phe-Met
Leu-enkephalin Tyr-Gly-Gly-Phe-Leu

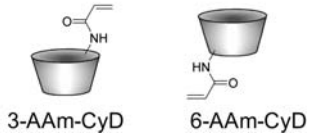

Figure 1. Templates, guests and vinyl monomers used in the present study.

presented in Figure 1). The imprinting efficiency at different pH is systematically investigated using various combinations of β-CyD vinyl monomer, template and guest.

2. Experimental

2.1 Materials

γ-Endor was synthesised by the 9-fluorenylmethyloxycarbonyl (Fmoc) method, and purified by reversed-phase HPLC. Met–Enk and Leu–Enk were purchased from Bachem (Bubendorf, Liestal, Switzerland). Water was purified by a Millipore Milli-XQ purification system (Millipore, Tokyo, Japan). β-CyD and *N*,*N*′-methylenebisacrylamide (MBAAm) were obtained from Wako Pure Chemical Industries, Ltd, Osaka, Japan. Silica gel used as a support was from Macherey-Nagel, Düren, 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.

2.2 Synthesis of vinyl monomers of CyD

6-AAm-CyD was prepared from mono[6-*O*-(*p*-toluenesulphonyl)]-β-CyD, obtained by reacting β-CyD with *p*-toluenesulphonyl chloride in dehydrated pyridine (*35*). This tosylate was converted to mono-6-amino-6-deoxy-β-CyD according to a previous report (*36*). Then, mono-6-amino-6-deoxy-β-CyD (1 g, 882 μmol) was added to DMF (10 ml). After the resulting solution became homogeneous, a mixture of acrylic acid (62 μl, 903 μmol) and *O*-(benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (334 mg, 881 μmol) in DMF (2 ml), as well as *N*,*N*-diisopropylethylamine (300 μl, 2.1 mmol), was added dropwise. The resultant mixture was stirred at room temperature overnight, and was evaporated. The crude solid product was purified by column chromatography on a Sephadex G-15 column (from Amersham Biosciences, Little Chalfont, UK) with water as the eluent to give a white product (0.11 g, 92.6 μmol) in 11% yield: matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF-MS, positive mode): obsd 1210.9 (calcd for $[M + Na^+]$: 1210.4). ^{1}H NMR (D_2O, 500 MHz): $\delta = 6.19$ (dd, 1H, $J = 10.3$, 16.9 Hz), 6.09 (d, 1H, $J = 16.5$ Hz), 5.68 (d, 1H, $J = 10.4$ Hz), 5.0–4.9 (m, 7H), 4.0–3.5 (m, 40H), 3.33 (t, 1H, $J = 9.2$ Hz) and 3.21 (dd, 1H, $J = 9.2$, 13.8 Hz).

3-AAm-CyD was synthesised according to a previous report (*28*). MALDI-TOF-MS (positive mode): obsd 1211.3 (calcd for $[M + Na^+]$: 1210.4). ^{1}H NMR (D_2O, 500 MHz): $\delta = 6.16$ (m, 2H), 5.69 (d, 1H, $J = 9.6$ Hz), 5.0–4.9 (m, 7H), 4.23 (m, 1H), 4.10 (bs, 1H) and 3.9–3.5 (m, 40H).

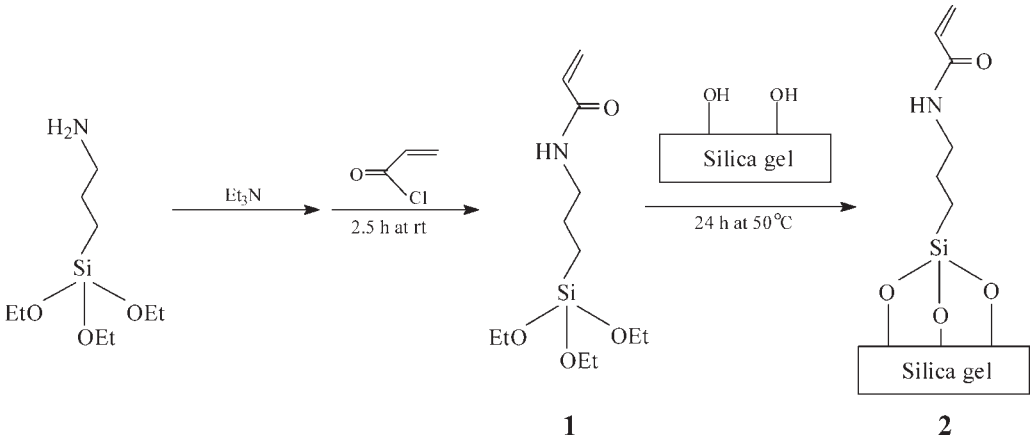

Figure 2. Introduction of acrylamido groups to silica gel.

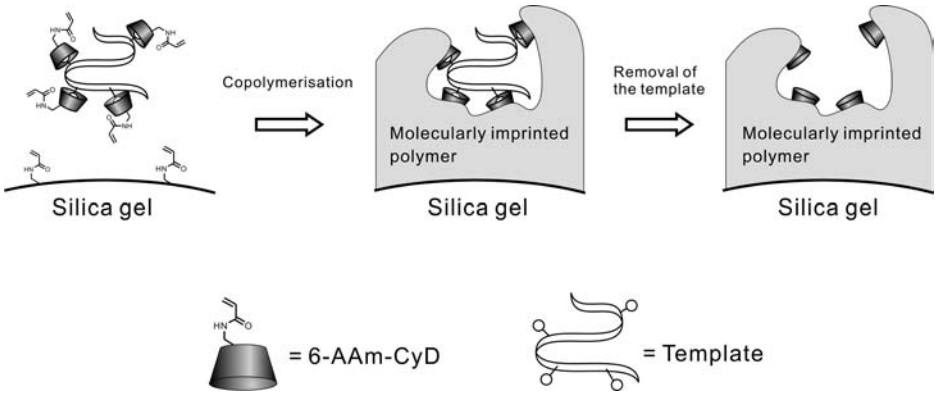

Figure 3. Scheme of molecular imprinting on the surface of silica gel.

2.3 *Introduction of acrylamido groups onto the surface of silica gel*

The synthetic route is given in Figure 2. Into 300 ml of chloroform, 3-aminopropyltriethoxysilane (10 ml, 45.2 mmol) and triethylamine (13 ml, 93.3 mmol) were poured. After the mixture was cooled with ice, acryloyl chloride (3.7 ml, 45 mmol) was added and stirred at room temperature for 2.5 h. After adding methanol (20 ml), the reaction solution was evaporated. The crude solid product was purified by silica column chromatography with chloroform/methanol (90:10 in v/v) as the eluent to give **1** (6.0 g) in 48% yield. Then, **1** (250 μl) was dissolved in 200 ml of xylene, and the silica gel (10 g), dried as described above, was added to this mixture. After stirring the mixture at 50°C for 24 h, the product was washed twice with water and methanol, respectively, and then dried *in vacuo* (the yield of **2** was 8.1 g). According to the titration with $KMnO_4$, about 140 μmol of vinyl groups were introduced to 1 g of the silica gel.

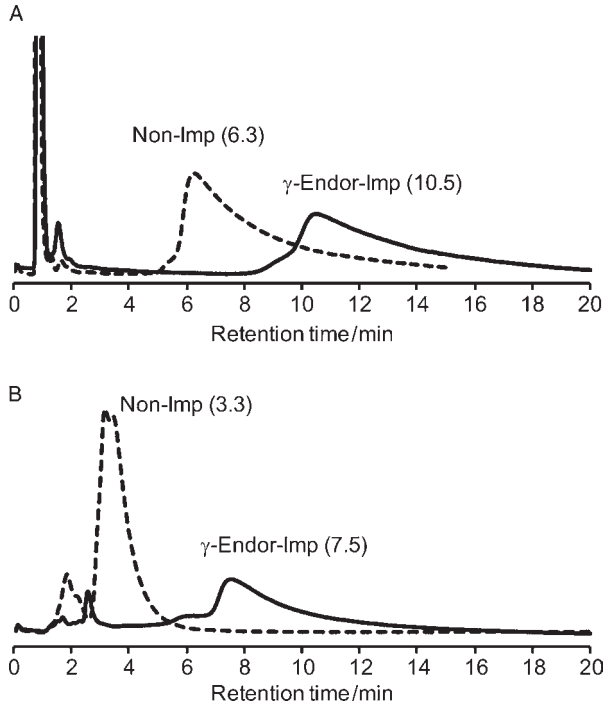

Figure 4. HPLC chromatograms for the γ-endor-imprinted polymer of 3-AAm-CyD (solid line) and the corresponding non-imprinted 3-AAm-CyD polymer (dotted line). Both the polymer preparation and the binding assay were achieved at (A) pH 3.0 and (B) pH 8.0. Column length = 50 mm; flow rate = 0.5 ml min^{-1}. The retention times are presented in parentheses. The peaks eluting before the main peaks are due to the impurities in the specimen of γ-endor. Imprinting conditions: the vinylated silica gel (600 mg), vinyl monomer of β-CyD (30 μmol), γ-endor (10 μmol), MBAAm (180 μmol), potassium persulphate (3 mg), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (3 μl) in 5 ml of buffer solution at 35°C for 20 h under nitrogen.

2.4 *Molecular imprinting on the surface of silica gel*

The typical reaction procedures were as follows. To the vinylated silica gel (600 mg) in 2 ml of buffer solution, the vinyl monomer of β-CyD (35.7 mg, 30 μmol), template oligopeptide (10 μmol) and MBAAm (27.7 mg, 180 μmol) as the cross-linking agent in 3 ml of buffer solution were added. The mixture was gently stirred at 35°C under nitrogen for 5 min. Then, potassium persulphate (3 mg, 11 μmol) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (3 μl, 20 μmol) were added. The mixture was further incubated at 35°C under nitrogen for 20 h. The product was washed with 100 ml of water (thrice) and subsequently with 100 ml of methanol (thrice).[1] The non-imprinted polymer (as the control) was prepared exactly in the same manner, except for the absence of the template.

2.5 *Evaluation of the efficiency of molecular imprinting by high-performance liquid chromatography (HPLC)*

The polymer/silica-gel composite thus obtained was directly packed in a stainless steel column tube (50 mm × 4.6 mm i.d., purchased from GL Science, Japan). The retention

Table 1. Retention factors k for the γ-endor-imprinted polymers of 3-AAm-CyD and 6-AAm-CyD prepared at pH 3.0 and 8.0.[a,b]

	3-AAm-CyD		6-AAm-CyD	
Guest	k_{imp}	k_{non}	k_{imp}	k_{non}
Imprinting at pH 3.0				
γ-Endor	10.3	6.1	11.8	7.0
Met–Enk	0.50	0.37	1.7	0.65
Leu–Enk	0.56	0.54	2.7	0.97
Imprinting at pH 8.0				
γ-Endor	3.7	0.92	2.7	2.6
Met–Enk	0.19	0.69	1.5	3.2
Leu–Enk	0.26	0.79	1.9	4.4

[a] Imprinting conditions: the vinylated silica gel (600 mg), vinyl monomer of β-CyD (30 μmol), γ-endor (10 μmol), MBAAm (180 μmol), potassium persulphate (3 mg), *N,N,N′,N′*-tetramethylethylenediamine (3 μl) in 5 ml of buffer solution. At 35°C for 20 h under nitrogen.
[b] The binding activity was assayed at the pH where the imprinting was carried out.

behaviour of the guest was monitored at 260 nm with the HPLC system (Jasco, Tokyo, Japan). The amounts of guest compound injected for HPLC were kept constant at 20 nmol. The flow rate of the eluent (50 mM Tris buffer at pH 8 or 50 mM citrate buffer at pH 3) was 0.5 ml min^{-1}. Prior to the analysis of imprinting efficiency, sufficient amount of the eluent was passed through the column until the baseline became flat. The activity of the imprinted polymers to bind a guest molecule ('binding activity') was evaluated in terms of the retention factor (k), calculated by $(t - t_0)/t_0$. Here, t and t_0 are the retention times of the guest and acetone (as the void marker), respectively. When β-CyD polymers were prepared independently, these k values were reproduced within ±10%.

2.6 Circular dichroism (CD) measurements

The CD spectra of the oligopeptides (0.004 mM) were measured on a JASCO J-820 spectropolarimeter at pH 8.0 (0.5 mM Tris buffer) or pH 3.0 (0.5 mM citrate buffer) at 25°C. The absorbance of all the samples was less than 1.4. Under these conditions, the Beer's law satisfactorily held, and reliable and reproducible CD signals were obtained.

3. Results and discussion

In the present imprinting, thin layers of the imprinted β-CyD polymer were immobilised on the surface of porous silica-gel supports, which were used as the stationary phase for HPLC (Figure 3). Unless noted otherwise, the guest-binding activities of the imprinted polymers were assayed at the pH where the imprinting reactions were carried out. Typical HPLC chromatograms are presented in Figure 4. Here, mono-3-(*N*-acrylamido)-3-deoxy-β-CyD (3-AAm-CyD) bearing an acrylamido residue in the secondary hydroxyl side of the cavity was imprinted towards γ-endor in either pH 3.0 citrate buffer or pH 8.0 Tris buffer. The retention time of the heptadecapeptide was remarkably increased, in comparison with the column of the non-imprinted polymer prepared in the absence of γ-endor, confirming clear-cut imprinting effects. The retention factors obtained by these analyses are summarised in Tables 1 and 2.

3.1 Molecular imprinting of β-CyD monomers towards γ-endor at pH 3.0

In the imprinting of 3-AAm-CyD towards γ-endor in pH 3.0 citrate buffer (50 mM), the retention factor k_{imp} of this imprinted polymer for γ-endor was 10.3, which was 1.7 times as large as that ($k_{non} = 6.1$) of the non-imprinted polymer (Table 1). On the other hand, the binding activities of this polymer to Met–Enk and Leu–Enk were hardly changed by this imprinting ($k_{imp} = 0.50$ and 0.56, whereas $k_{non} = 0.37$ and 0.54, respectively). The k_{imp} for γ-endor is 21 and 18 times as large as those of Met–Enk and Leu–Enk, respectively. Thus, the molecular recognition of this polymer towards γ-endor is highly strict.

Table 2. Retention factors k for the Met–Enk- or Leu–Enk-imprinted polymers of 3-AAm-CyD and 6-AAm-CyD prepared at pH 3.0 and 8.0.[a]

	3-AAm-CyD			6-AAm-CyD		
Guest	Met–Enk-imp	Leu–Enk-imp	Non-imp	Met–Enk-imp	Leu–Enk-imp	Non-imp
Imprinting at pH 3.0						
γ-Endor	5.0	4.1	6.1	10.1	10.6	7.0
Met–Enk	0.36	0.32	0.37	0.42	0.48	0.65
Leu–Enk	0.53	0.49	0.54	0.67	0.70	0.97
Imprinting at pH 8.0						
γ-Endor	0.96	1.4	0.92	0.51	0.99	2.6
Met–Enk	0.11	0.20	0.69	1.8	1.0	3.2
Leu–Enk	0.17	0.26	0.79	2.4	1.7	4.4

[a] The binding activity was assayed at the pH where the imprinting was carried out. The conditions for the imprinting are presented in the footnote of Table 1.

Apparently, strong binding sites for γ-endor were formed during the imprinting process.[2] This remarkable discrimination of γ-endor from Met–Enk and Leu–Enk indicates that this imprinted polymer does not bind only the N-terminal portion of γ-endor (note that Met–Enk is the N-terminal pentapeptide of γ-endor and Leu–Enk is its $Met^5 \rightarrow Leu^5$ homologue). The Thr^6–Leu^{17} portion of γ-endor is certainly taking primary roles in the binding to the imprinted polymer, although the N-terminal pentapeptide could also be bound by the polymer [Tyr^1, Phe^4 and Met^5 (or Leu^5) residues have intrinsically strong binding affinity to β-CyD (*9*)].

In the molecular imprinting of mono-6-(*N*-acrylamido)-6-deoxy-β-CyD (6-AAm-CyD) towards γ-endor at pH 3.0, the imprinting effect was also remarkable (Table 1). This β-CyD monomer possesses a vinyl group in the primary hydroxyl side of the cavity. The retention factor k for the binding of γ-endor increased from 7.0 to 11.8 by the imprinting, whereas the binding of Met–Enk and Leu–Enk by this imprinted polymer was only weak. Thus, β-CyD polymers that selectively bind γ-endor have been successfully prepared by the molecular imprinting of 3-AAm-CyD and 6-AAm-CyD at pH 3.0.

3.2 Molecular imprinting of β-CyD monomers towards γ-endor at pH 8.0

The molecular imprinting towards γ-endor was also successful at pH 8.0 (50 mM Tris buffer) when 3-AAm-CyD was used as the functional monomer (Table 1). The binding of γ-endor was greatly promoted by the imprinting ($k_{imp}/k_{non} = 4.0$), whereas the bindings of Met–Enk and Leu–Enk were notably weakened ($k_{imp}/k_{non} = 0.3$ and 0.3, respectively). Apparently, the binding sites that are specific to γ-endor were formed in the polymer during the imprinting. As discussed above, the C-terminal region of γ-endor, as well as its N-terminal region, could be recognised by the β-CyD moieties.

With the use of 6-AAm-CyD as the functional monomer at pH 8.0, however, the imprinting was not very effective (Table 1). The binding activity of the imprinted polymer towards γ-endor was almost the same as that of the non-imprinted polymer. At this pH, the non-imprinted β-CyD polymer prepared from 6-AAm-CyD also strongly bound Met–Enk and Leu–Enk. These non-specific bindings were also notable for the γ-endor-imprinted polymer, although they were suppressed by the imprinting.[3] As a result, the differentiation of γ-endor by the polymer from Met–Enk and Leu–Enk was poor.

3.3 Molecular imprinting of β-CyD monomers towards Met–Enk and Leu–Enk

The imprinted polymers, prepared from 3-AAm-CyD and 6-AAm-CyD using Met–Enk or Leu–Enk as the template at both pH 3.0 and 8.0, showed almost no selective recognition towards the corresponding template molecule (Table 2). For example, the retention factor k_{imp} (for Met–Enk) of the Met–Enk-imprinted polymer, prepared from 3-AAm-CyD at pH 3.0, was 0.36, which was almost the same as that ($k_{non} = 0.37$) of the corresponding non-imprinted polymer. Similarly, the binding activity towards the template was decreased by the imprinting, when 3-AAm-CyD was imprinted to Met–Enk or Leu–Enk at pH 8.0 or when 6-AAm-CyD was imprinted at pH 3.0 or 8.0. Met–Enk and Leu–Enk are inappropriate for the template of the present imprinting. These results are consistent with the above conclusion that the N-terminal portion of γ-endor is not the sole binding target of γ-endor-imprinted β-CyD polymers.

3.4 Effects of conformations of the oligopeptides on the molecular imprinting

Figure S1(A) (see Supporting Information) shows CD spectra of γ-endor. At both pH 3.0 and 8.0, negative broad CD signals were observed in the amide absorption region from 200 to 230 nm, indicating an almost completely random structure. This result is consistent with the fact that β-endorphin has little, if any, secondary structure at pH 5.9 (*37*) (the sequence of γ-endor is identical with the 17 amino acid residues in the N-terminus of β-endorphin). In these random structures, apolar moieties of γ-endor [Thr^6, Thr^{12}, Pro^{13}, Leu^{14}, Val^{15}, Thr^{16} and Leu^{17}, in addition to Tyr^1, Phe^4 and Met^5 (or Leu^5)] can sufficiently interact with β-CyD molecules in the imprinting solutions, since they are rather free from intramolecular hydrophobic aggregations. According to our previous study (*30*), the imprinted β-CyD polymers memorise peptide conformations rather than their primary structures alone. In the CyD polymers imprinted to the oligopeptides, several CyD molecules are immobilised complementarily to the apolar and bulky groups of the template. Thus, the structural and physico-chemical properties of the template are precisely and vividly memorised in terms of the positions and orientations of the CyD molecules. On the other hand, both Met–Enk and Leu–Enk have weak but explicit positive ellipticities in 200–230 nm (Figure S1(B,C)). According to the detailed analysis of the CD spectra (*38*), these apolar pentapeptides retain some organisation and have ordered structures in water. It is inferred that these side-chain groups show notable mutual interactions in these conformations and thus their interactions with β-CyD molecules in the imprinting solutions are inefficient.

3.5 Comparison of 3-AAm-CyD and 6-AAm-CyD as monomers for molecular imprinting

As shown in Table 1, the imprinting of 3-AAm-CyD towards γ-endor is highly successful at both pH 3.0 and

8.0 ($k_{imp}/k_{non} = 1.7$ and 4.0, respectively). The imprinting increased the binding of only γ-endor and suppressed (or little changed) the binding of Met–Enk and Leu–Enk. As a result, high selectivity to γ-endor has been obtained (k_{imp}(γ-endor)/k_{imp}(Met–Enk) = 21 and k_{imp}(γ-endor)/k_{imp}(Leu–Enk) = 18 at pH 3.0; k_{imp}(γ-endor)/k_{imp}(Met–Enk) = 19 and k_{imp}(γ-endor)/k_{imp}(Leu–Enk) = 14 at pH 8.0). However, the imprinting of 6-AAm-CyD to γ-endor is successful only at pH 3.0 and never at pH 8.0. Even at pH 3.0, the bindings towards Met–Enk and Leu–Enk were notably increased by the imprinting ($k_{imp}/k_{non} = 2.6$ and 2.8, respectively; see Table 1), and thus guest selectivity is poor (k_{imp}(γ-endor)/k_{imp}(Met–Enk) = 6.9 and k_{imp}(γ-endor)/k_{imp}(Leu–Enk) = 4.4, respectively). In order to recognise the structure of γ-endor strictly, 3-AAm-CyD that bears a vinyl group in the secondary hydroxyl side of the cavity is superior to 6-AAm-CyD, as was proposed in a previous paper (*28*). With the use of 3-AAm-CyD as the functional monomer, the polymerisation proceeds near the guest molecule in the imprinting process so that the positions and orientations of β-CyD residues in the imprinted polymers are more strictly and precisely regulated. Note that, in the formation of CyD inclusion complexes, benzene, phenol and other guest molecules preferentially enter the cavity of CyD from its secondary hydroxyl side (*9*). On the other hand, the polymerisation of 6-AAm-CyD occurs rather far away from the guest molecule, making the memory of the template faint. Furthermore, the 6-methylene group between the acrylamido group and the β-CyD in 6-AAm-CyD provides larger flexibility for the movements of the β-CyD molecules in the polymers, and this factor further makes the guest recognition less strict.

4. Conclusion

In the present paper, molecular imprinting of vinyl monomers of β-CyD to γ-endor, an opioid oligopeptide, has been achieved in bulk water. In the process of the imprinting, several CyD molecules are placed and fixed complementarily to the apolar groups of the oligopeptide template. Thus, only the binding to γ-endor is promoted by the cooperation of these CyD molecules, whereas the binding to relevant peptides Met–Enk and Leu–Enk is suppressed by the imprinting. The guest selectivity is high especially when a vinyl group is introduced to the secondary hydroxyl side of the cavity of CyD (k_{imp}(γ-endor)/k_{imp}(Met–Enk) = 21 and k_{imp}(γ-endor)/k_{imp}(Leu–Enk) = 18). These results indicate that imprinting of CyD is promising to prepare artificial receptors for peptides and proteins. These attempts, as well as more detailed study on the mechanism of molecular recognition by the imprinted polymers, are currently under way in our laboratory.

Acknowledgements

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Notes

1. The solutions with which the molecularly imprinted polymer was washed (300 ml of water + 300 ml of methanol) were analysed by HPLC. It was confirmed that at least 60–80% of the template molecules were removed from the polymer by the washing procedure employed here.
2. As the injection amount of γ-endor in the HPLC analysis increases, its retention time gradually decreases. This result may indicate that the binding sites in the imprinted polymers prepared here are not necessarily very abundant. In order to compare the binding activities of various guest molecules precisely, the amounts of sample injected were, throughout the present paper, kept constant at 20 nmol as described in Section 2.
3. Non-specific binding of Met–Enk and Leu–Enk is significant only with this polymer (this effect is marginal for any of the non-imprinted 6-AAm-CyD polymers prepared at pH 3.0, non-imprinted 3-AAm-CyD polymers prepared at pH 8.0 and non-imprinted 3-AAm-CyD polymers prepared at pH 3.0).

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