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Original Article

Efficient Separation of Hydrophobic Molecules by Molecularly Imprinted Cyclodextrin Polymers

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Abstract

Cyclodextrins were cross-linked with toluene 2,4-diisocyanate in dimethyl sufoxide in the presence of hydrophobic biomolecules as templates, and the imprinted polymers were applied to the stationary phases of high performance liquid chromatography. Molecular imprinting efficiently promoted the binding-affinity and substrate-selectivity towards the template molecule, compared with the control polymers prepared in their absence. When cholesterol (template molecule) was complexed with cyclodextrins prior to the polymerization, for example, the imprinted polymer retained cholesterol more strongly than other steroids. Upon the polymerization without a template molecule, the binding towards steroids was much weaker. Besides steroids, imprinting was effective for various hydrophobic and rigid template molecules. Since binding of the guest molecule was based on inclusion complex formation with cyclodextrins, separation could be achieved in the solvents containing water. These polymeric receptors are also applicable to selective recognition of biologically important molecules or removal of toxic molecules from aqueous media. Thus, imprinting of cyclodextrins is useful for the preparation of synthetic tailor-made receptors for various kinds of hydrophobic guest molecules.

Introduction

Various molecular assemblies which can recognize specific guest molecules have been proposed to mimic antibodies and enzymes [1]. These organic assemblies were usually constructed from calixarenes [2], cyclophanes [3], and cyclodextrins (CyDs) [4]. For example, dimers and trimers of CyDs that are connected with appropriate linkers efficiently recognize large guest molecules such as steroids, peptides, and other biologically important molecules [5]. Although these host assemblies are very effective for molecular recognition, there still remains a wide gap between naturally occuring receptors and artificial ones. One of the promising methodologies to fill this gap is molecular imprinting [6]. This technique is based on (1) adduct formation between functional monomer(s) and a template molecule, (2) immobilization of the system by polymerization, and (3) removal of template molecules from the polymer (see Figure 1). By changing the template molecule, versatile artificial receptors that recognize specific molecule can be easily synthesized in a tailor-made fashion [7].

Recently, CyD assemblies were successfully prepared by using molecular imprinting technique as schematically illustrated in Figure 1, and hydrophobic steroids were efficiently bound by these imprinted polymers in aqueous media. From batch-wise experiments, imprinted CyD polymers, prepared in the presence of cholesterol, bound cholesterol (the template) much more efficiently and selectively than did random CyD assemblies prepared in its absence [8, 9]. According to physicochemical analysis, guest-binding sites were composed of two or three CyD molecules [9].

Here, we apply these molecularly imprinted CyD polymers to high performance liquid chromatography (HPLC) stationary phases. It is shown that highly specific recognition towards the template molecule is attained when this imprinted polymer is packed into HPLC column. Predominance of molecularly imprinted CyD polymers for the separation in water-based media is clearly demonstrated.

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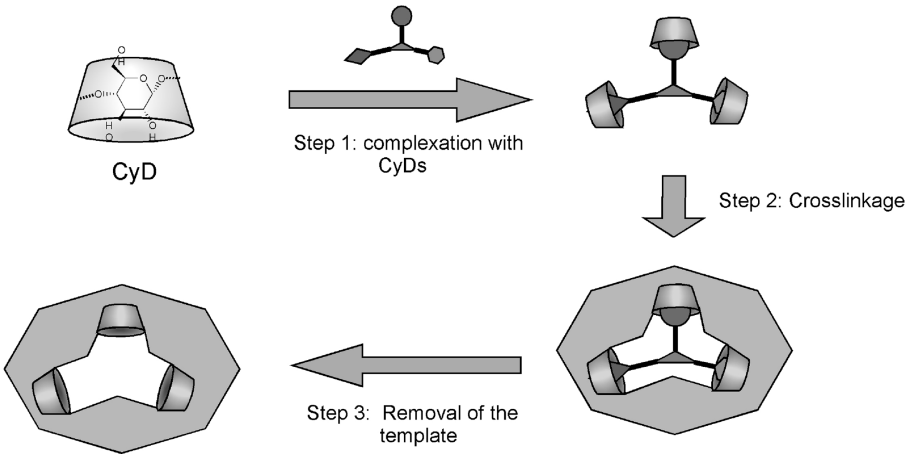

Figure 1. Strategy of the molecular imprinting of cyclodextrins (CyDs) for the preparation of tailor-made receptors.

Experimental

Materials

All the reagents (CyDs, steroids as well as other hydrophobic guests, cross-linking agents, and others) were purchased from Tokyo Kasei Co. Ltd. Dimethyl sulfoxide (DMSO) was dried with molecular sieve 4A and then distilled under a reduced pressure. Steroids and CyDs were dried *in vacuo* at 70 °C for 24 h before use. Water was purified by a Millipore Milli-XQ purification system. A stainless steel column (4.6 mm × 5.0 cm) and packer (50 mL) were purchased from GL Science Co. Ltd.

Preparation of imprinted CyD polymers and HPLC stationary phases

β-CyD was cross-linked with toluene 2,4-diisocyanate (TDI) as reported before [9a]. In dry DMSO, β-CyD (88 mM) was treated with the diisocyanate (560 mM unless otherwise noted) in the presence of a template (44 mM). After being magnetically stirred at 65 °C for 2 h, the gel formed was chopped into pieces, washed with acetone, and ground with mortar and pestle. The polymer was sufficiently washed with acetone, tetrahydrofuran (THF), and hot water to remove the template molecule, CyDs, and the cross-linker. Then, the polymer was dried *in vacuo* at 70 °C for 24 h. Non-imprinted CyD polymers (control polymers) were prepared in the same manner as described above except for the absence of template molecules. The polymer obtained was further finely ground with mortar and pestle, and then particles with the size between 38 and 63 μm were collected by using stainless sieves. The powder was added to an aqueous acetonitrile (AN) solution (AN/water = 50/50 (v/v)) and suspended by an ultrasonic sonicator for 10 min. After being incubated for 10 min, the supernatant of the suspension was removed. This sonication–suspension procedure was repeated three times, and then the residue was packed into a stainless steel column by using a stainless packer (50 mL). The control (non-imprinted) polymer, which was prepared in the absence of the template, was similarly treated and packed into a column.

Measurement of retention times of substrates

The column obtained was first eluted with acetonitrile until the base line became stable. A mixture of water and acetonitrile was used as eluent, and the retention times were measured by a UV detector ($\lambda = 220$ nm for steroids and 260 nm for aromatic compounds) [10]. Flow rate was 0.5 mL/min and acetone was used as a void marker. The retention times, measured twice, were identical with each other within 0.1 min [11]. 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. Imprinting efficiency was estimated from the ratio of k_{imp} with respect to k_{non} (k_{imp}/k_{non}), where k_{imp} and k_{non} are capacity factors of the imprinted and non-imprinted polymers, respectively [6].

Results and discussion

Remarkable imprinting of β-CyD for the recognition of hydrophobic guests

The presence of cholesterol as a template during the polymerization significantly affected the retention behaviors towards the steroids family listed in Figure 2. When the polymer was prepared in the absence of template, the capacity factor for cholesterol was only 0.1 (see Table 1). Thus, cholesterol was hardly retained by

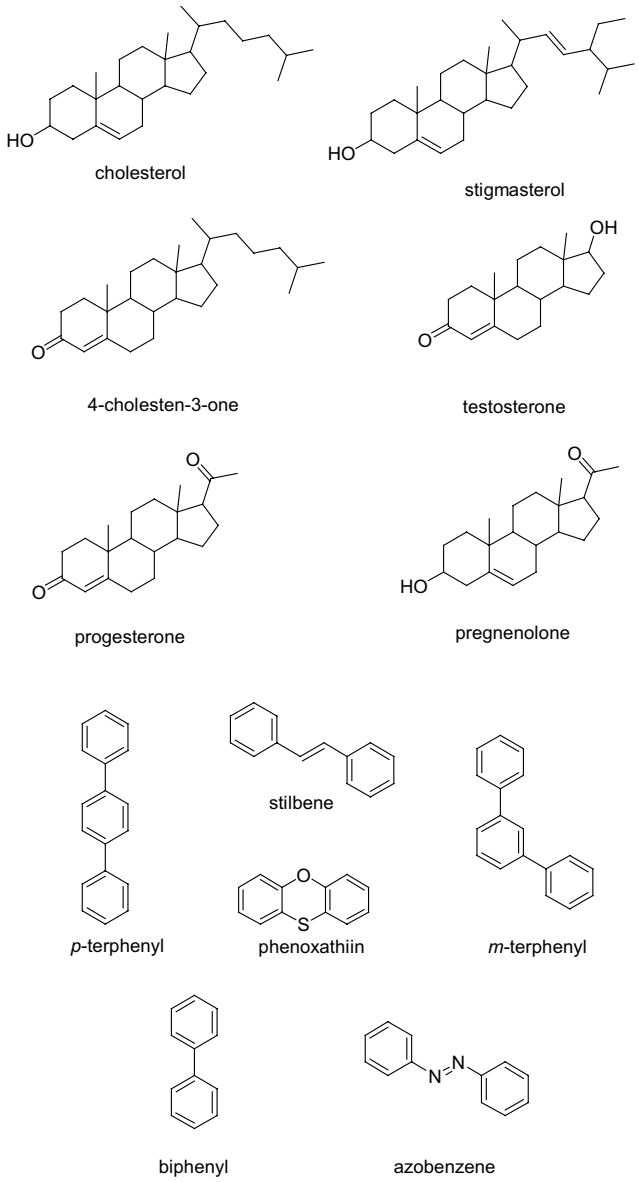

Figure 2. Structures of steroids used as templates and guests in the present study.

Table 1. Capacity factors of cholesterol-imprinted and non-imprinted β-CyD polymers cross-linked with TDI

Guests	Capacity factor k[a]		k_{imp}/k_{non}
	k_{imp}	k_{non}	
Cholesterol	2.05	0.10	21
Stigmasterol	0.59	0.42	1.4
Prognenolone	1.97	0.50	3.9
Progesterone	1.20	0.69	1.7
4-Cholesten-3-one	0.52	0.20	2.6

[a] Eluent: water/AN = 5/95 (v/v).

this non-imprinted polymer. On the contrary, the capacity factor dramatically increased from 0.1 to 2.05 when the polymerization was carried out in the presence of cholesterol [11]. The imprinting efficiency (k_{imp}/k_{non}) for cholesterol was as high as 21. With other steroids as substrates, however, capacity factors of this cholesterol-imprinted polymer were much smaller. It should be noted that the capacity factors for 4-cholesten-3-one and stigmasterol were not much promoted by the cholesterol-imprinting, although they have similar structures to cholesterol [12]. These facts demonstrate that imprinting of β-CyD provides specific binding sites for the template in the polymer. Similar results were obtained upon the use of stigmasterol as a template in place of cholesterol: the capacity factor for stigmasterol was improved from 0.42 to 2.5 under the same HPLC conditions employed. When α-CyD was used as a host molecule instead of β-CyD, however, the column obtained did not retain the steroids at all (data not shown). This result clearly showed that guest molecules are bound to the β-CyD assemblies formed by the imprinting, not to the cavity formed by the cross-linking molecules in the polymers.

Table 2. Retention behavior of hydrophobic rigid guests

Polymer (template)	Capacity factor k		
	trans-stilbene	*m*-terphenyl	*p*-terphenyl
P(*trans*-stilbene)	**1.9**	0.99	0.66
P(*m*-terphenyl)	0.83	**1.6**	0.56
P(*p*-terphenyl)	0.99	0.85	**2.0**

t_0: retention time of acetone. Eluent: water/AN = 25/75 (v/v).

Besides steroid family, distinct imprinting effect was also observed as long as the template molecules had enough rigidity and hydrophobicity. Shape of the hydrophobic molecule could be discriminated by the imprinting as depicted in Table 2: the polymer imprinted with *p*-terphenyl retained the template (*p*-terphenyl) most strongly among three hydrophobic molecules tested here. As expected, *m*-terphenyl- and *trans*-stilbene-imprinted β-CyD polymers selectively and preferentially bound the corresponding template molecules, respectively. Complex formation of β-CyDs with these rigid templates contributes to the imprinting effect [13].

Dependence of water content in the eluent on the capacity factor

Since the main driving force for the binding of the guest molecule is hydrophobic interaction for the inclusion complex formation, the presence or absence of water in the eluent significantly affected the capacity factor. As shown in Figure 3, the improvement of the capacity factor by the imprinting is evident only when the eluent for the analysis involves water (see the rows of 5/95 and 10/90 (v/v) in Figure 3). With the use of AN 100% as eluent where hydrophobic interaction did not much work, however, no improvement of the capacity factor for cholesterol was observed. Under these conditions, hydrogen bonding would rather dominate the retention

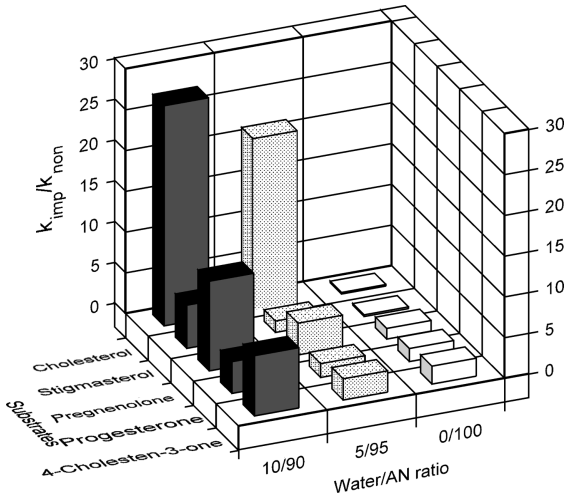

Figure 3. Effect of water content in eluent on the imprinting efficiency (k_{imp}/k_{non}).

behavior because imprinting efficiency for the guest molecules depends on the number of hydrogen-bonding sites in each molecule. In water/AN = 5/95 or 10/90 (v/v), where hydrogen bonding is destroyed by the competition with water and hydrophobic interaction is dominant, selective recognition toward cholesterol was attained. Thus, the imprinting effect of cholesterol can be attributed to inclusion complex formation, not to hydrogen bonding or other interactions. Selectivity towards the cholesterol decreased with decreasing AN content in eluent, due to the improvement of the capacity factors for steroids other than cholesterol (compare black bars with gray bars in Figure 3). Since strong binding sites for cholesterol dominate the retention behavior at relatively high content of AN, the imprinted polymer exhibited high selectivity towards the cholesterol. In contrast, weak binding sites as well as strong ones participate in the recognition at a lower content of AN, and thus the selectivity is decreased.

Optimum concentration of TDI for the imprinting

In order to prepare cross-linked β-CyD polymer which is strong enough for the application to the stationary phases of HPLC, the TDI/β-CyD ratio should be more than 4.0. With lower ratio of TDI/CyD than 4.0, no gels were obtained in the solutions upon the imprinting polymerization. However, increase in the TDI concentration ([TDI] = 1120 mM) significantly interfered the imprinting. As shown in Table 3, imprinting efficiency was rather small (compare Table 1 with Table 3) and substrate-selectivity of the polymers toward cholesterol against stigmasterol was almost nil. High cross-linkage ratio would restrict the flexibility which is essential for the selective binding and lower the selectivity. Therefore, optimum TDI/β-CyD ratio for the effective imprinting was around 6.

Selective recognition of phenoxathiin (a dioxin analogue)

Dioxin is one of the most toxic molecules that are to be removed from the environments. However, this compound does not have effective functional groups available for precise molecular recognition (such as hydrogen bonding sites), which makes selective binding of dioxin rather difficult (see phenoxathiin in Figure 2 as a dioxin analogue) [14]. Due to the lack of hydrogen bonding sites, application of conventional molecular imprinting is also difficult. On the contrary, dioxin should be a good template for the present imprinting because it is based on the hydrophobic inclusion complexation of CyDs with hydrophobic rigid templates. Therefore, we applied this technique to the recognition of dioxin analogue, phenoxathiin [14]. As expected, retention time for phenoxathiin was greatly enhanced by the imprinting as shown in Figure 4 (from 3.7 to 7.3 min, while retention times of other molecules were essentially unchanged). With this imprinted CyD polymer, separation of phenoxathiin from other molecules is possible. For example, the mixtures of phenoxathiin and *p*-terphenyl could not be separated with non-imprinted polymer, because the retention times of these compounds were similar (1.8 min for *p*-terphenyl and 3.7 for phenoxathiin). But the imprinting enhanced the difference of retention times of these molecules (1.8 min for *p*-terphenyl and 7.3 min for phenoxathiin) and facilitated their separation (data not shown). The present imprint-

Table 3. Capacity factors of cholesterol-imprinted and non-imprinted β-CyD polymer prepared with high concentration of TDI[a,b]

Guests	Water/AN = 20/80		k_{imp}/k_{non}	Water/AN = 40/60		k_{imp}/k_{non}
	Capacity factor			Capacity factor		
	k_{imp}	k_{non}		k_{imp}	k_{non}	
Cholesterol	1.1	0.40	2.8	8.1	3.1	2.6
Stigmasterol	1.2	0.45	2.7	9.7	3.6	2.6
Pregnenolone	1.1	0.53	2.1	2.1	1.6	1.3
Progesterone	1.1	0.90	1.2	3.2	2.6	1.2
4-Cholesten-3-one	1.1	0.90	1.2	10.8	4.9	2.2

[a] [TDI] = 1120 mM ([TDI]/[β-CyD] = 12.7).
[b] Retention time was very short in eluent with high content (>90%) of AN.

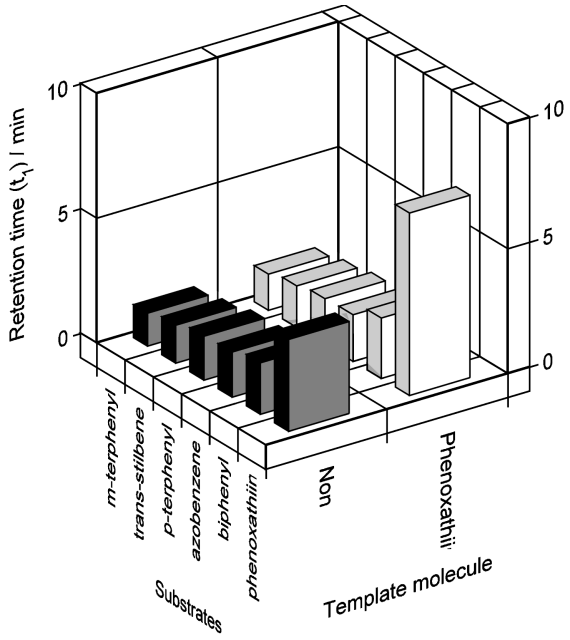

Figure 4. Binding of various guests by the imprinted β-CyD polymers obtained with the use of phenoxathiin (an analogue of dioxin) as the template.

ing is applicable to even the templates which show no hydrogen-bonding interaction.

Conclusions

(1) β-Cyclodextrin was cross-linked with toluene 2,4-diisocyanate in dimethyl sufoxide in the presence of hydrophobic molecules as templates, and the polymers were successfully applied to the stationary phases of high performance liquid chromatography.
(2) Molecular imprinting of β-CyD efficiently promoted the binding-affinity and substrate-selectivity towards the template molecules compared with the control polymers. By using this technique, cholesterol was efficiently and selectively recognized among the steroids family. In the present imprinting, hydrophobic interaction, which is usually non-selective, could be used for selective and cooperative recognition.

This method has an advantage in preparing artificial receptors for the chemicals which are sparingly soluble in water. Besides CyDs, modified CyDs and other host molecules are promising candidates as recognition sites for still more efficient and selective artificial receptors in a tailor-made fashion.

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10. At a higher ratio of water in the eluent, peaks were too broadened to determine retention times precisely.
11. The peaks were rather broad as observed in the conventional imprinting. However, the retention times could be precisely determined.
12. These chromatographic results are consistent with those obtained from batch-wise adsorption assay reported in [9].
13. NOE was observed between CyD molecules and the guest (*p*-terphenyl) in DMSO, indicating that inclusion complex was formed during polymerization. UV spectrum was also changed (peak shift was observed) on addition of β-CyD in DMSO.
14. Phenoxathiin is similar in structure to dioxin compounds except for the lack of halogen atoms.