Molecularly Imprinted Cyclodextrins as Selective Receptors for Steroids[1]

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ABSTRACT: β-Cyclodextrin (β-CyD) was cross-linked by toluene 2,4-diisocyanate in the presence of various steroids in dimethyl sulfoxide, and the steroids were removed after the polymerization. The molecularly imprinted β-CyD polymer, obtained by using cholesterol or stigmasterol as the template, efficiently and reversibly bound the steroid in the mixtures of water and tetrahydrofuran. In these polymers, the mutual orientation of β-CyD molecules is regulated so that they cooperatively bind the target guests which are too large to be included in the cavity of one β-CyD molecule. When either the alkyl residue at the 17-position or the hydroxyl residue at the 3-position is absent from the template, however, the molecular imprinting is much less efficient. The mechanism for the imprinting and the structure of the guest-binding sites in the imprinted polymers have been proposed.

Introduction

In nature, biopolymers such as proteins, DNAs, and saccharides precisely recognize their counterparts in water and, as the result, give rise to the desired functions. Mimicking this molecular recognition through chemical procedures is one of the greatest challenges. A number of receptors for small guests (less than a few angstroms in size) have been already synthesized, and the importance of the physicochemical complementarity between host and guest has been concluded.[2] However, the design of receptors for large guest molecules is far more difficult, since several functional groups and apolar sites must be placed precisely at the predetermined and distant positions. There still remains a gap between the chemically obtained molecular-recognition systems and naturally occurring ones.[3]

The methodology we propose here to overcome this problem is to prepare ordered assemblies of cyclodextrin (CyD) by using the molecular imprinting method (see Figure 1).[1,4,5] CyDs are cross-linked in the presence of a large guest, and the mutual orientation of the CyD molecules in the CyD−guest complex is immobilized in the polymeric structures. Although each of the CyD molecules binds only a part of the guest, the ordered assemblies as a whole recognize the large guest very selectively. Steroids used here as the guests are too large to be accommodated in the cavity of one CyD molecule, and two (or more) CyD molecules must function cooperatively to bind them efficiently.[6−8]

This paper reports on the selective binding of steroids in aqueous media by the ordered assemblies of β-CyD.[9] Dependencies of the imprinting efficiency on the molecular structures of template and cross-linking agent, as well as on the reaction conditions, are clarified. Furthermore, the structures of guest-binding sites in these polymers are analyzed in terms of the relationship between the guest structure and the binding activity.

Experimental Section

Materials. The chemical structures of the guests are presented in Figure 2. 1-Phenylazonaphthalene was prepared according to the literature,[10] and the others were purchased from Tokyo Kasei Co. β-CyD and the steroids were dried in vacuo at 40 °C, immediately before the polymerization. Dimethyl sulfoxide (DMSO) and pyridine were dried with molecular sieve 4A and distilled under a reduced pressure. Water was purified by a Millipore Milli-XQ purification system.

Preparation of Imprinted β-CyD Polymers. Most of the imprinted polymers were prepared by the following "one-step method". This method takes advantage of in situ formation of the β-CyD−steroid complex in the polymerization mixtures. β-CyD (4.4 mmol) and a steroid as the template (1.5 mmol unless noted otherwise) were dissolved in dry DMSO (50 mL), and toluene 2,4-diisocyanate (TDI, 28 mmol) was added. 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 hot water, tetrahydrofuran (THF), and hot ethanol to remove the template molecule, β-CyD, and TDI. Then the polymer was dried in vacuo at 40 °C for 24 h. The urethane linkages in the polymers were shown by ^{13}C-CP/MAS NMR spectroscopy. The NMR spectroscopy further confirmed that the template molecules were completely removed from the polymers by the treatments with the solvents (data not presented). Thus, the 3-OH of cholesterol (and of other steroids) did not react with the TDI during the polymerization, to a measurable extent. (Under the reaction conditions, the OH residues of β-CyD are in more than 60-fold excess to the OH of cholesterol and preferentially react with TDI.) The mole ratios of TDI to β-CyD in the imprinted polymers were evaluated by elemental analysis (e.g., the ratio for the cholesterol-imprinted polymer was 3.4). In the "two-step method", the 3:1 β-CyD/cholesterol complex was first prepared by adding aqueous solution of β-CyD to hexane solution of cholesterol.[6] The complex was isolated and sufficiently dried in vacuo and then treated with TDI in dry DMSO.

As a control, nonimprinted polymers were prepared by cross-linking β-CyD with TDI in the absence of the templates. The reaction conditions and the methods of polymer treatments were the same as described for the imprinted polymers.

The ^{1}H NMR spectra of the polymerization mixtures containing β-CyD and cholesterol were measured in DMSO at 65 °C on a JEOL 270 MHz NMR spectrometer, by using 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid as the internal standard.

Determination of Guest-Binding Activities of the β-CyD Polymers. The β-CyD polymer (0.5 mmol in β-CyD residue) was incubated for 1 h with stirring at 25 °C in 11 mL of water/THF mixture (5/6 in volume) containing 0.05 mmol

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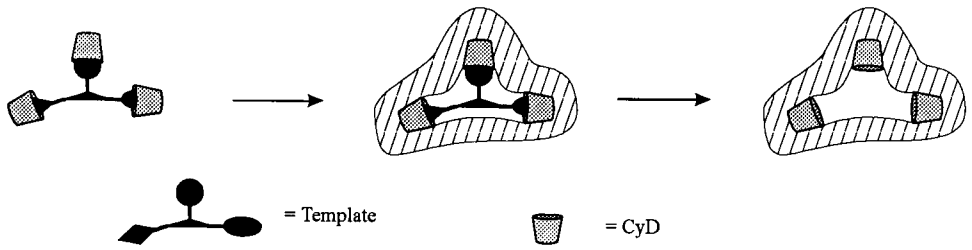

Figure 1. Molecular imprinting of CyD for the preparation of ordered assemblies as tailor-made receptors.

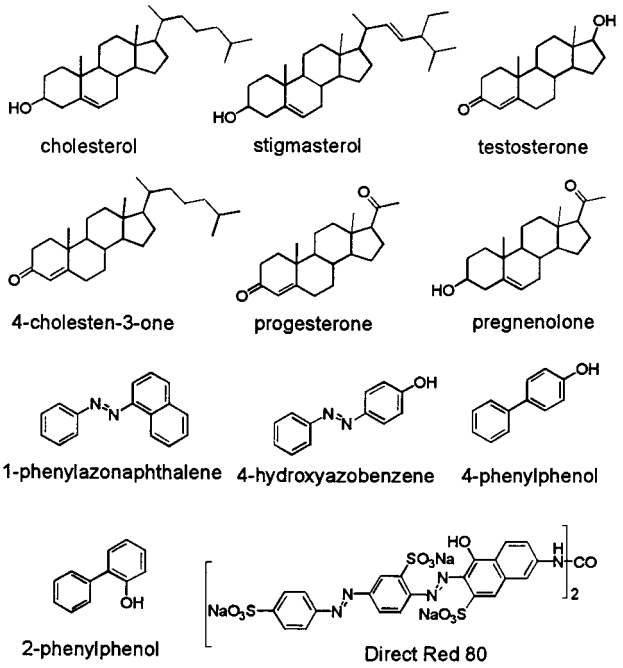

Figure 2. Chemical structures of steroids and other guests used in this study.

of steroid. In this solvent, the steroids were completely soluble, but the polymers were insoluble. The equilibrium for the guest binding was attained within 10 min. Then, the mixture was centrifuged, and the concentration of steroid in the liquid phase was determined by gas chromatography (Shimadzu GC-8A; a Silicon G-17 glass column (3.2 mm × 2 m)). The mole fraction of the guest bound by the polymer (with respect to the total amount of guest) was taken as the guest-binding activity of the polymer.

The binding of aromatic guests by the polymeric receptors was carried out in 5/6 water/methanol mixtures. The concentration of the unbound guest was determined by either reversed phase HPLC (for phenol; with a Merck LiChrosphere RP-18(e) ODS column) or UV/vis absorption spectroscopy (for others; on a JASCO V-520 spectrometer).

Evaluation of the Magnitude of Imprinting Effect. The magnitude of molecular imprinting effect was evaluated in terms of the "imprinting-induced promotion of binding" (IPB). This value is defined by

$$\mathrm{IPB} = (A_{\mathrm{imp}} - A_{\mathrm{non-imp}})/A_{\mathrm{non-imp}} \quad (1)$$

Here, A_{imp} is the amount of the guest that was bound by the imprinted polymer under the conditions described above, and $A_{\mathrm{non-imp}}$ is the corresponding value for the nonimprinted polymer. The IPB values reflect the efficiency of the imprinting more correctly than do the A_{imp}'s themselves, since the difference in the intrinsic binding activities of various guests toward β-CyD residues is normalized.

Results

Reversible Binding of Cholesterol by Cholesterol-Imprinted β-CyD Polymers. The imprinted β-CyD polymer, prepared in the presence of cholesterol (by the "one-step method"), efficiently and promptly adsorbed cholesterol in water/THF mixtures. Under the conditions described in the Experimental Section, 70% of the cholesterol was bound by the polymer in the equilibrium. (More than 85% of the equilibrium amount was adsorbed within 1 min.) The corresponding equilibrium value for the nonimprinted polymer was 34%. The "imprinting" significantly promotes the binding of cholesterol. When the adduct between the β-CyD polymer and cholesterol was treated with boiling ethanol, all the cholesterol in the adduct was promptly released to the liquid phase. On the following cholesterol-binding experiment, both the rate of binding and the capacity were identical with those in the foregoing run. The present guest binding is completely reversible.

Table 1. Potentials of Various Steroids as Templates for the Molecular Imprinting[a]

	binding activity toward the template molecule (%)[b]		
template	imprinted	nonimprinted	IPB (%)[c]
cholesterol	70	34	110
stigmasterol	92	48	92
pregnenolone	33	30	10
4-cholesten-3-one	61	58	5
testosterone	13	23	−43
progesterone	24	36	−33

[a] The polymers were prepared by using TDI, and the template molecule was used as the guest for each of the imprinted polymers. [b] The mole fraction of the guest bound by the polymer in the 5/6 water/THF mixture, with respect to the total amount of guest. Detailed experimental conditions are described in the Experimental Section. [c] The IPB value is defined by eq 1.

The imprinted β-CyD polymer, obtained by the "two-step method", showed virtually the same rate and capacity in the guest-binding as the one obtained by the "one-step method". Thus, all the other imprinted polymers were synthesized by the "one-step method".

Dependence of the Magnitude of Imprinting Efficiency on the Structure of Steroid Template. When stigmasterol was used as the template, the molecular imprinting was also successful (IPB = 92%, Table 1). On imprinting with either 4-cholesten-3-one (without the OH residue at the 3-position) or pregnenolone (without the apolar alkyl chain at the 17-position) as the template, however, the binding of the corresponding template molecule by the polymer was only marginally promoted. Furthermore, the binding activities toward testosterone and progesterone, which have neither the 3-OH nor 17-alkyl residues, were diminished by imprinting the polymers with them. Thus, both of these residues in the steroid templates are required for effective imprinting.

Selectivities of the Imprinted β-CyD Polymers in Steroid Binding. The selectivities for the binding of steroids by the imprinted polymers are presented in

Table 2. Binding Activities of the Cholesterol-Imprinted and the Stigmasterol-Imprinted β-CyD Polymers toward Various Steroids at 25 °C

guest	cholesterol-imprinted		stigmasterol-imprinted	
	binding activity[a]	IPB (%)[b]	binding activity[a]	IPB (%)[b]
cholesterol	70 (34)	110	36 (34)	6
stigmasterol	82 (48)	71	92 (48)	92
4-cholesten-3-one	83 (58)	43	63 (58)	9
testosterone	31 (23)	35	15 (23)	−35
progesterone	39 (36)	8	24 (36)	−33

[a] The mole fraction of the guest bound by the polymer in the 5/6 water/THF mixture. The numbers in the parentheses show the activities of the nonimprinted polymer. [b] The IPB value is defined by eq 1.

Table 2. As expected, the stigmasterol-imprinted polymer shows a high selectivity toward stigmasterol (see the right-hand-side column). Although the nonimprinted polymer binds all the steroids in considerable amounts (the numbers in parentheses), only the binding of stigmasterol is greatly enhanced by the molecular imprinting. The IPB value for the stigmasterol binding is 92%, whereas those for the binding of cholesterol and 4-cholesten-3-one are only 6 and 9%. The binding toward testosterone and progesterone is suppressed by the imprinting. As a result, stigmasterol is quite selectively bound by the imprinted polymer.

The selectivity for the cholesterol binding is also improved by imprinting with cholesterol (the middle column in Table 2). Here, stigmasterol and 4-cholosten-3-one are also significantly bound, since the nonimprinted polymer intrinsically prefers them, and this preference cannot be overridden with the imprinting. However, the imprinted polymer discriminates between the steroids with the 17-alkyl residues and the ones without them. (Cholesterol is bound more efficiently than are testosterone and progesterone.) The structures of the templates are satisfactorily memorized in these imprinted polymers. Note that all these selectivities are accomplished by a batchwise operation. The guest selectivities would be much higher if the columns, packed with these polymeric receptors, are used for the separation.[11]

Reaction Conditions Required for the Efficient Imprinting. The amount of cholesterol bound by the cholesterol-imprinted polymer monotonically increased with increasing $[\text{cholesterol}]_0/[\beta\text{-CyD}]_0$ ratio in the polymerization mixtures, up to the ratio of 1 (the open circles in Figure 3). In contrast, the binding of phenol was not at all enhanced by the imprinting, irrespective of the ratio (the closed circles). This result rules out the possibility that the "imprinting effect" is simply associated with the increase in the amount of the β-CyD being available for the guest binding.

Use of DMSO as the polymerization solvent is advantageous for the present imprinting. When the polymerization was achieved in a 1:1 DMSO/pyridine mixture, for example, the imprinting effect was nil. Other organic solvents investigated did not sufficiently dissolve β-CyD. As the cross-linking agent, hexamethylene diisocyanate was also available. The amount of the cholesterol bound by the imprinted polymer was 15%, whereas that of the nonimprinted polymer was less than 1%. When β-CyD was cross-linked by epichlorohydrin in aqueous alkaline solutions, however, the polymer did not bind cholesterol. The β-CyD inclusion complex is hardly formed under these conditions, making the imprinting ineffective.[1,4]

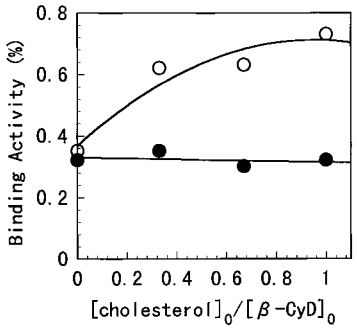

Figure 3. Mole fraction of the guest, bound by the cholesterol-imprinted β-CyD polymer at 25 °C, as a function of $[\text{cholesterol}]_0/[\beta\text{-CyD}]_0$ ratio in the polymerization mixtures: the guests are cholesterol (○) and phenol (●). The conditions for the binding are shown in the Experimental Section.

Table 3. Binding Activities of the Cholesterol-Imprinted β-CyD Polymer toward Various Aromatic Guests at 25 °C

guest	molecular size (Å)[a]	IPB (%)[b]	binding activity (%)[c]
4-hydroxyazobenzene	11	44	23 (16)
1-phenylazonaphthalene	10	29	44 (34)
4-phenylphenol	8	5	68 (65)
2-phenylphenol	7	4	58 (56)
phenol	4	9	35 (32)
Direct Red 80	40		0 (0)

[a] The length of guest molecule (the distance between the two ends). [b] The IPB value is defined by eq 1. [c] The mole fraction of the guest bound by the polymer in the 5/6 water/methanol mixture. The numbers in parentheses show the activities of the nonimprinted polymer.

Complex Formation between β-CyD and Cholesterol in the Reaction Mixtures. When β-CyD was added to a DMSO solution of cholesterol, the NMR signal of the 18-methyl protons of the cholesterol gradually shifted toward the higher magnetic field. The upfield shift was 0.040 ppm, when $[\text{cholesterol}]_0 = 2$ and $[\beta\text{-CyD}]_0 = 50$ mM at 65 °C. These NMR shift changes satisfactorily fit the curve for the formation of the 1:1 β-CyD−cholesterol complex. (This result is consistent with the finding by Breslow et al. that the 1:1 complex is formed in water.[7]) The complex formation constant in DMSO at 65 °C, determined from the curve, is 550 M^{-1}. More than 98% of the β-CyD in the present polymerization mixtures forms the complex with cholesterol.

Assessment of the Structure of Guest-Binding Sites in the Cholesterol-Imprinted β-CyD Polymer. To shed light on the structure of the guest-binding sites, the magnitudes of the imprinting effects, induced by the cholesterol template, on the binding of various aromatic guests were analyzed (Table 3). The IPB value directly reflects the imprinting-induced increase in the number of binding sites for each of the guests. The amounts of the bound guests themselves are inappropriate as the indexes for the purpose, since they are primarily governed by the intrinsic affinities of the guests to β-CyD. All of the guests used here are so large that several β-CyD molecules are required to accommodate them completely. Thus, the guest binding should be notably promoted, if two (or more) β-CyD molecules are appropriately located in the polymer and cooperatively bind the guest.

The binding of 4-hydroxyazobenzene and 1-phenylazonaphthalene is strengthened by the imprinting (IPB = 44 and 29%, respectively). Apparently, the orientation

of β-CyD molecules in the cholesterol-imprinted polymer is suitable for the cooperative binding of these large guests. However, the binding of 2-phenylphenol and 4-phenylphenol, which are smaller in size, is only faintly enhanced (IPB = 4 and 5%). The IPB value for phenol is also small. Direct Red 80, a large dye molecule, is not adsorbed at all by the polymer. These results will be discussed later in terms of the structure of the guest-binding sites.

Discussion

Formation of the Imprinted β-CyD Polymers. The 1:1 β-CyD−cholesterol complex is essential for the present imprinting. Thus, the imprinting is successful in DMSO, where the CyD inclusion complexes are formed,[4] but not in the DMSO/pyridine mixtures. In the latter solvent system, the complex formation is competitively inhibited by the pyridine molecules. The "one-step method" and the "two-step method" provide virtually the same imprinting effects, since the β-CyD−cholesterol complex, formed in situ, is responsible for the imprinting.

The polymerization involves a stepwise cross-linking of β-CyD molecules. First, the β-CyD in the β-CyD−cholesterol complex reacts with one of the two isocyanate residues in a TDI molecule, forming a urethane linkage. Here, only a part of the cholesterol molecule is accommodated in the cavity of the β-CyD, and the rest, which is also apolar, is still contacting with DMSO. Thus, another β-CyD molecule is bound to this apolar moiety of the cholesterol, and in this complex, the OH residue of the second β-CyD reacts with the other isocyanate residue of the TDI molecule. As the result, these two β-CyD molecules are connected by two urethane linkages. In a similar way, a number of β-CyD molecules are cross-linked with each other, and the structure of the template is memorized in their mutual conformation. Consistently, the cholesterol-binding activity monotonically increases, even when the $[\text{cholesterol}]_0/[\beta\text{-CyD}]_0$ ratio is greater than 1/3 (see Figure 3). If the imprinted polymer were to be formed by the cross-linking of the 3:1 β-CyD−cholesterol complex (or of 2:1 complex if any), the binding activity should attain the maximum at the ratio 1/3 (or 1/2) and not increase anymore.

The imprinting by cholesterol is far more efficient than that by 4-cholesten-3-one (Table 1). Assumedly, the 3-OH of cholesterol forms a hydrogen bond with the OH residue of β-CyD, regulating the depth of penetration of the guest in the cavity of the β-CyD. The alkyl residues at the 17-position are essential for the imprinting, since they are included in the cavity of one of the two β-CyD molecules that form the guest-binding sites (vide infra).

Guest-Binding Site in the Imprinted β-CyD Polymers. The results in Table 3 indicate that the guest-binding site is mainly composed of two β-CyD molecules that are connected by one TDI molecule.[12] According to the study using a CPK molecular model, the distance between the rims of the two β-CyDs in this structure is about 7 Å. When 4-hydroxyazobenzene or 1-phenylazonaphthalene interacts with this dimeric β-CyD, each of the two aromatic residues of the guest is accommodated in the cavity of one of the two β-CyDs (Figure 4b).[7,13] The cooperation of the two β-CyDs takes place, although the penetration of the aromatic rings in the cavities is rather shallow. In contrast, the binding of 2- and 4-phenylphenols is weak, since the two β-CyDs are too far away from each other to show a cooperative binding toward these smaller guests (c).

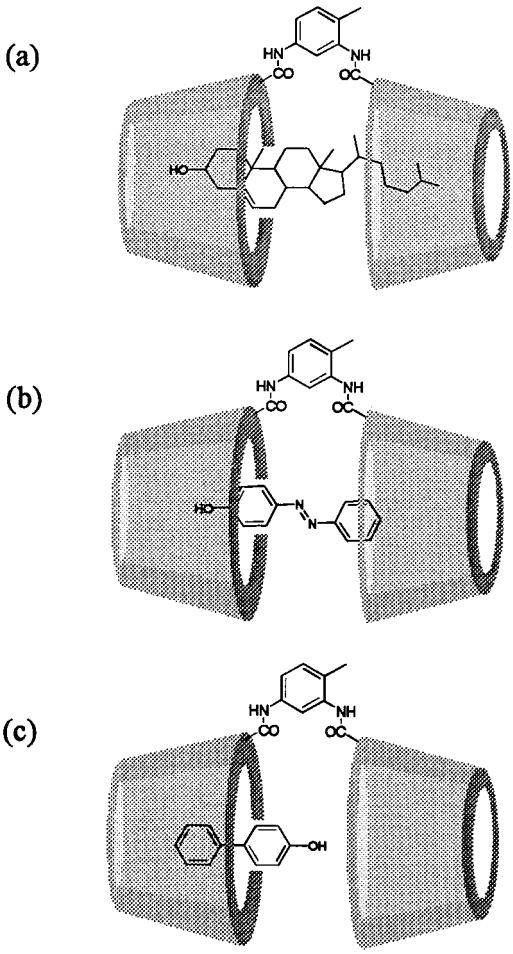

Figure 4. Binding modes of (a) cholesterol, (b) 4-hydroxyazobenzene, and (c) 4-phenylphenol to the guest-binding sites in the cholesterol-imprinted β-CyD polymer.

On the basis of these arguments, the binding mode of cholesterol to the imprinted β-CyD polymers is proposed as depicted in Figure 4a. The left-hand side of the cholesterol molecule is included in the cavity of one β-CyD, whereas the 17-alkyl residue is in the cavity of another β-CyD.[7,14] In the imprinted polymers, these guest-binding sites are highly abundant, as clearly indicated from the polymerization mechanism. The proposed binding mode is consistent with the fact that the steroids having 17-alkyl residues (cholesterol, stigmasterol, and 4-cholesten-3-one) are bound more strongly than those without the residues (testosterone and progesterone). The 3-OH of steroids plays only a minor role in the binding to the polymers (Table 2), despite its notable effect in the imprinting. In aqueous media (for the binding), the hydrogen bonding of this residue with the OH residue of β-CyD should be less efficient than in DMSO (for the imprinting).

In conclusion, polymeric receptors, which show great binding activities and high selectivities toward steroids, are prepared by using molecular imprinting technique. The guest binding takes place in aqueous media and is reversible, and thus these receptors are potential for industrial application (the removal of cholesterol from dairy products,[15] therapeutic uses, and others). The present method should be useful for the preparation of

various receptors, especially toward large guests of nanometer sizes.

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