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Spectroscopic Anatomy of Molecular-Imprinting of Cyclodextrin. Evidence for Preferential Formation of Ordered Cyclodextrin Assemblies

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Abstract: The processes of molecular-imprinting of β-cyclodextrin (β-CyD) with cholesterol and stigmasterol (cross-linking agent = diisocyanate) have been analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy. These templates enormously promote the formation of dimers and trimers of β-CyD, which are only inefficiently formed in their absence. These ordered assemblies are the guest-binding sites, in which two or three β-CyD molecules cooperate to bind large steroids. Ordered assemblies are also formed when 2,6-di-*O*-methyl-β-cyclodextrin is used in place of β-CyD. Direct spectroscopic evidence for molecular-imprinting effect has been obtained. Molecular imprinting of CyDs is potent for tailor-made preparation of synthetic receptors for nanometer-scaled guests.

Introduction

Recent progresses in host−guest chemistry are so remarkable that selective hosts can be obtained as long as the target guest is small in size.[1−5] Cyclodextrins (CyDs),[6] crown ethers,[7] calixarenes,[8] and others,[9] as well as their chemically modified derivatives, are typical examples. However, design of artificial receptors for nanometer-scaled guests is far more difficult, and has not yet been sufficiently accomplished. These receptors are regarded as one of the keys for the future science and technology, where a wide spectrum of large molecules (e.g., proteins, nucleic acids, polysaccharides, and bioactive materials) must be strictly differentiated from each other.

One of the most promising candidates is ordered assemblies of host molecules which recognize small guests.[10−12] When these hosts are placed complementarily to a large guest and each of them binds a predetermined portion of the guest, the assembly as a whole recognizes this large guest very precisely. In previous papers,[13−15] the authors showed that the molecular-imprinting method[16−23] is useful in preparing ordered assemblies of hosts in tailor-made fashion. For example, CyD polymers imprinted with cholesterol selectively and efficiently bound cholesterol.[13,14] It was proposed that CyD molecules were regularly aligned and

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cooperatively bound the target guest. Consistently, nonimprinted CyD polymers involving random orientation of CyDs hardly bound cholesterol.

In this paper, the processes of molecular-imprinting of β-CyD with steroids are analyzed in detail by mass spectroscopy and ^{1}H NMR. Preferential formation of dimers and trimers of β-CyD during the imprinting processes is directly shown, and all the species in the imprinting mixtures are characterized. These ordered assemblies are the guest-binding sites in the molecularly imprinted β-CyD polymers. These arguments are further supported by use of 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CyD) in place of β-CyD. The imprinting mechanism and the roles of templates are discussed in terms of all these results.

Results

Mass Spectroscopy on the Molecularly Imprinted Mixtures. In the presence of steroid templates, β-CyD was reacted with cross-linking agent in dimethyl sulfoxide (DMSO) at 65 °C for 2 h, and water-soluble specimens were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOFMS). Accordingly, all the products formed during the molecular-imprinting processes have been clarified.[24,25] The structures of the chemicals used are presented in Figure 1.

Molecular-Imprinting by Use of Toluene 2,4-Diisocyanate (TDI) as Cross-Linking Agent. Figure 2a shows the mass spectrum for the reaction mixture obtained with cholesterol as the template. Under these imprinting conditions, dimers of β-CyD ($(CyD)_2$: mass number (M) = 2444−3184), as well as its trimers ($(CyD)_3$: M = 3927−4667), are efficiently formed. Monomeric β-CyDs ($(CyD)_1$: M = 1135−1727) are only minor products. Each of the signals in the dimer and trimer regions (also in the monomer region) corresponds to a different number of substitutions by TDI. For example, the signal at M = 2444 (the smallest molecule in the dimer region) is assigned to the β-CyD dimer in which two β-CyD molecules are bridged by one TDI molecule (**2**$^{(0)}$ in Figure 3). When one of the β-CyDs in this dimer further reacts with another TDI molecule, the mass number is increased to 2592 (the peak designated as **2**$^{(1)}$). The signal at M = 4223 represents the trimer (**3**$^{(2)}$) in which three β-CyDs are connected and two TDI molecules are tethered to these β-CyDs.[26] All the products of the molecular-imprinting

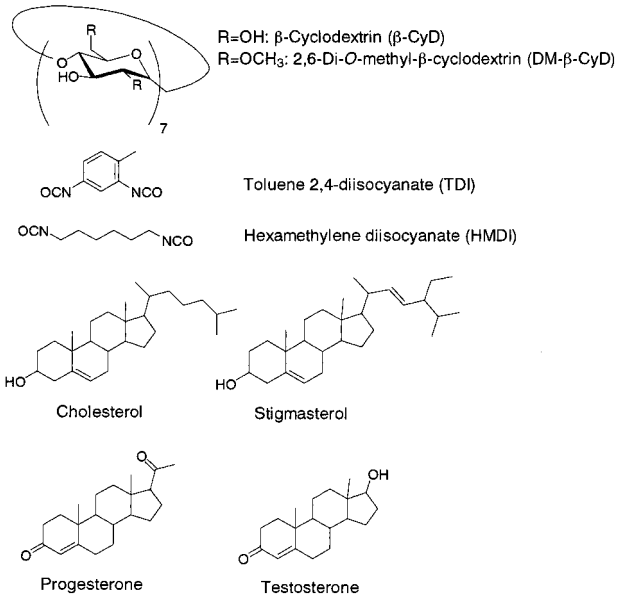

Figure 1. Chemical structures of β-CyD, DM-β-CyD, steroids, and cross-linking agents.

are presented in Figure 3, and the intensities of their mass signals are listed in Table 1. Under the present imprinting conditions, the most dominant species are β-CyD dimers bearing 2−4 TDI residues (**2**$^{(2)}$, **2**$^{(3)}$, and **2**$^{(4)}$, respectively). The second isocyanate group in these TDI substituents remains intact during the imprinting reactions.[27]

When stigmasterol was used in place of cholesterol, the mass spectra were similar to that in Figure 2a (data not presented). The formation of β-CyD dimers and trimers was also substantial. As the template for the molecular-imprinting of β-CyD, this steroid is as eminent as cholesterol and notably promotes the binding activity of the resultant β-CyD polymer (toward stigmasterol).[14]

When the reactions are achieved in the absence of cholesterol and stigmasterol, however, the mass spectra are dramatically changed (Figure 2b). Here, most of the products are monomeric β-CyDs (with a different number of TDI substitutions). Dimers of β-CyD are only marginal, and trimers are nil. It is concluded that these ordered assemblies of β-CyD are efficiently formed only in the presence of appropriate template. Direct spectroscopic evidence for the molecular-imprinting has been obtained.[28,29]

Under the imprinting conditions for Figure 2a, the total amount of β-CyD dimers is far greater than that of the intramolecularly bridged β-CyD (the species **1**$^{(intra)}$ in Figure 3). When one of the isocyanate groups of TDI reacts with β-CyD to form **1**$^{(1)}$, the second isocyanate group therein preferentially chooses the OH group of another β-CyD molecule, rather than another OH of the first β-CyD. Apparently, the intermolecular

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(24) The products were usually detected as the adducts with Na^+ or K^+. For the purpose of clarity, the mass numbers *M*, obtained after subtracting the values for these metal ions (23 and 39) from experimentally determined values, are presented in the text.
(25) Covalent cholesterol-β-CyD-TDI conjugates were not formed in detectable amounts.
(26) The major trimers involve three TDI molecules which are bridging three β-CyDs (see Figure 3). Thus, either of two pairs of β-CyDs is doubly bridged. In the β-CyD dimers, however, only singly bridged species were detected.
(27) These isocyanates are converted to amino groups by reacting with water in the posttreatment.
(28) Time-courses of mass intensity for the dimers are presented in Supplemental Figure 1.
(29) The dimers and trimers of β-CyD were selectively collected when the original imprinting products were treated with a membrane filter (see Experimental Section). The mass spectrum is shown in Figure 3 in the Supporting Information.

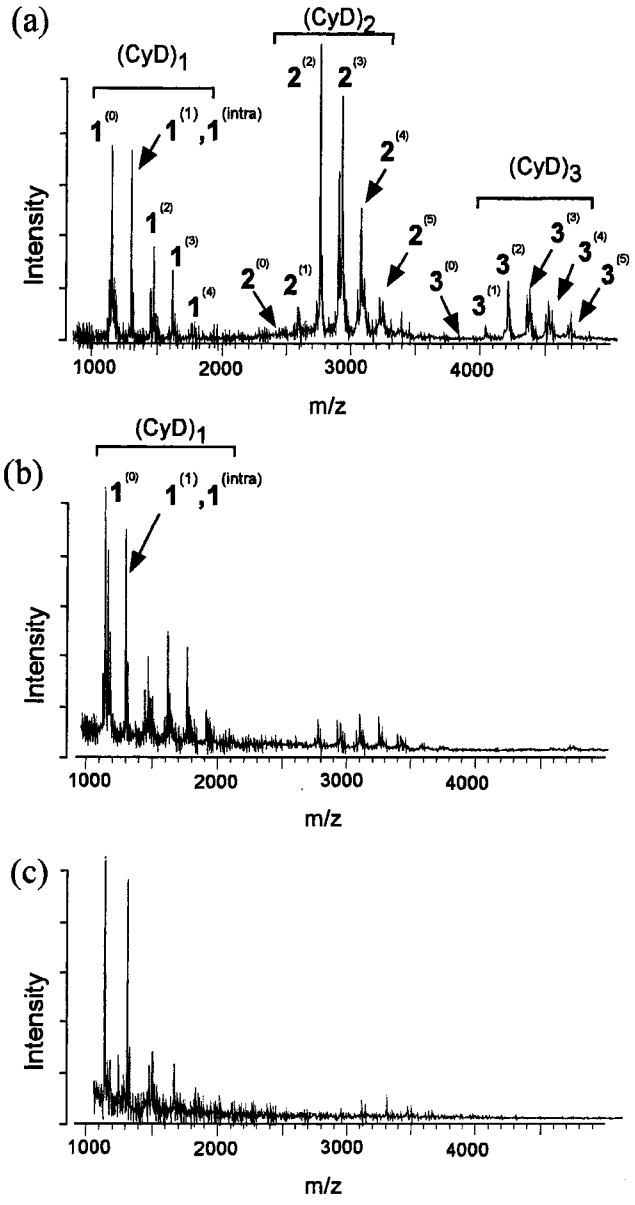

Figure 2. MALDI-TOFMS of TDI-cross-linked β-CyDs: (a) cholesterol-imprinted, (b) nonimprinted, and (c) progesterone-imprinted. [β-CyD] = 100 mM, [TDI] = 200 mM, and [cholesterol] = 50 mM; in dry DMSO at 65 °C for 2 h. The chemical structure for the corresponding species is presented in Figure 3.

reaction, which is otherwise far less efficient than the intramolecular one, is greatly promoted by the template. As described below, the second β-CyD forms an inclusion complex with the template, and is placed near the isocyanate group. The intrinsic reactivity of this isocyanate is not much changed, since $\mathbf{1}^{(intra)}$ is formed in a notable amount even in the absence of the template (Figure 2b).

Molecular-Imprinting by Use of Hexamethylene Diisocyanate (HMDI) as Cross-Linking Agent. The molecular-imprinting effect appears in mass spectra still more vividly when HMDI is the cross-linking agent (Figure 4). In the presence of cholesterol (a), dimeric β-CyDs ($(CyD)_2$) are produced in a notable amount (the multiple peaks are assignable to a different number of substitutions by HMDI). In the absence of the template (b), however, only monomeric β-CyDs ($(CyD)_1$: M = 1135−1845) are formed. Bridging of two β-CyD molecules

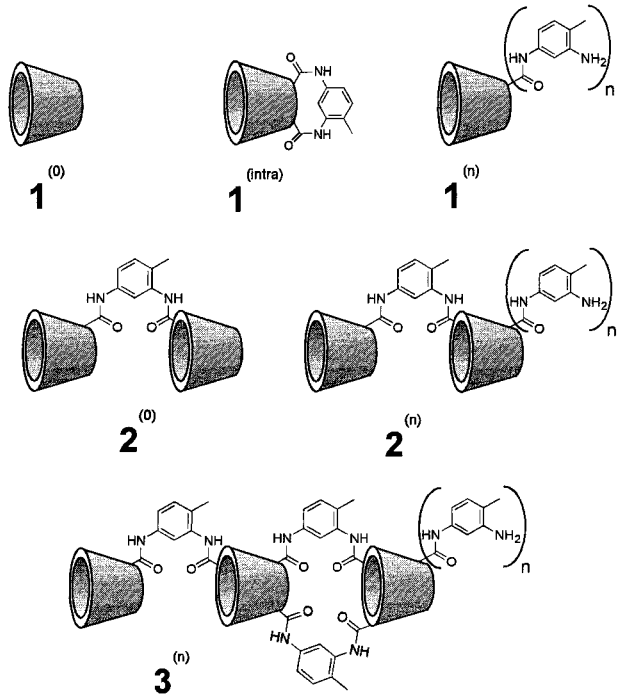

Figure 3. β-CyD-based species formed in the molecular-imprinting mixtures for Figure 2.

to dimers hardly occurs. These results are totally consistent with the previous results that only the imprinted β-CyD polymers efficiently bind cholesterol.[13,14] Without the molecular-imprinting, the formation of dimeric β-CyDs (the guest-binding sites) is difficult, mainly because the hexamethylene chain in HMDI is very flexible.

Mass Spectroscopy on the Reaction Mixtures Obtained with Ineffective Templates. Figure 2c shows the MALDI-TOFMS spectrum for the water-soluble mixture prepared from β-CyD and TDI in the presence of progesterone. This steroid, which has no long alkyl chain, is an ineffective template (the binding activity of β-CyD polymers toward progesterone is hardly improved by the imprinting with it).[14] Significantly, the mass spectrum for the progesterone-"imprinting" reaction is almost the same as that for the nonimprinting reaction (compare b and c in Figure 2). The formation of dimeric β-CyDs is never promoted. Essentially the same result was obtained when testosterone, another ineffective steroid template, was used. Thus, the mass spectroscopy fairly reflects the magnitude of imprinting efficiency (on guest-binding). *Only the agent that promotes the formation of β-CyD dimers and trimers is eminent for the molecular-imprinting.*[30]

^{1}H NMR Analysis on the Molecular-Imprinting Reactions. Figure 5 shows the time-courses for the reaction between β-CyD and TDI. In the absence of the cholesterol template (the open circles), only the primary OH groups of β-CyD react with TDI to form urethane linkages (b). The secondary ones, which are intrinsically less reactive, are kept almost intact (a). In the presence of cholesterol (the closed circles), however, even the

(30) The imprinting effect is dependent on the formation of 1:1 adduct between β-CyD and cholesterol. The binding constant of this adduct is 550 M^{-1} in DMSO-d_6 at 65 °C (ref 13b). The ROESY spectroscopy in DMSO-d_6 showed weak but explicit cross-peaks between the methyl protons of cholesterol and the protons of β-CyD. On the other hand, no NOE was observed for the progesterone-β-CyD system. It is indicated that both the steroid framework and the long alkyl chain of cholesterol are interacting with β-CyD in DMSO (progesterone has no long alkyl chain).

Table 1. Possible Structures of β-CyD-TDI Conjugates Assigned from MALDI-TOFMS

species	m/Z(M)	intensity[a] nonimp	intensity[a] imp	possible structure
CyD	1135	++++++++	+++++	**1**$^{(0)}$
CyD=TDI	1309	+++++++	+++++	**1**$^{(intra)}$
CyD-TDI	1283	+++	++	**1**$^{(1)}$
CyD-$(TDI)_2$	1431	+++	+++	**1**$^{(2)}$
CyD-$(TDI)_3$	1579	++++	++	**1**$^{(3)}$
CyD-$(TDI)_4$	1727	+++	+	**1**$^{(4)}$
CyD-TDI-CyD	2444	+	+	**2**$^{(0)}$
CyD-TDI-CyD-TDI	2592	+	++	**2**$^{(1)}$
CyD-TDI-CyD-$(TDI)_2$	2740	+	++++++++++	**2**$^{(2)}$
CyD-TDI-CyD-$(TDI)_3$	2888	++	++++++++	**2**$^{(3)}$
CyD-TDI-CyD-$(TDI)_4$	3036	+	+++++	**2**$^{(4)}$
CyD-TDI-CyD-$(TDI)_5$	3184	+	++	**2**$^{(5)}$
CyD-$(TDI)_2$-CyD-TDI-CyD[b]	3927		+	**3**$^{(0)}$
CyD-$(TDI)_2$-CyD-TDI-CyD-TDI	4075		+	**3**$^{(1)}$
CyD-$(TDI)_2$-CyD-TDI-CyD-$(TDI)_2$	4223		++++	**3**$^{(2)}$
CyD-$(TDI)_2$-CyD-TDI-CyD-$(TDI)_3$	4371		+++	**3**$^{(3)}$
CyD-$(TDI)_2$-CyD-TDI-CyD-$(TDI)_4$	4519		++	**3**$^{(4)}$
CyD-$(TDI)_2$-CyD-TDI-CyD-$(TDI)_5$	4667		+	**3**$^{(5)}$

[a] Relative intensity of the signal from Figure 2. [b] See ref 26.

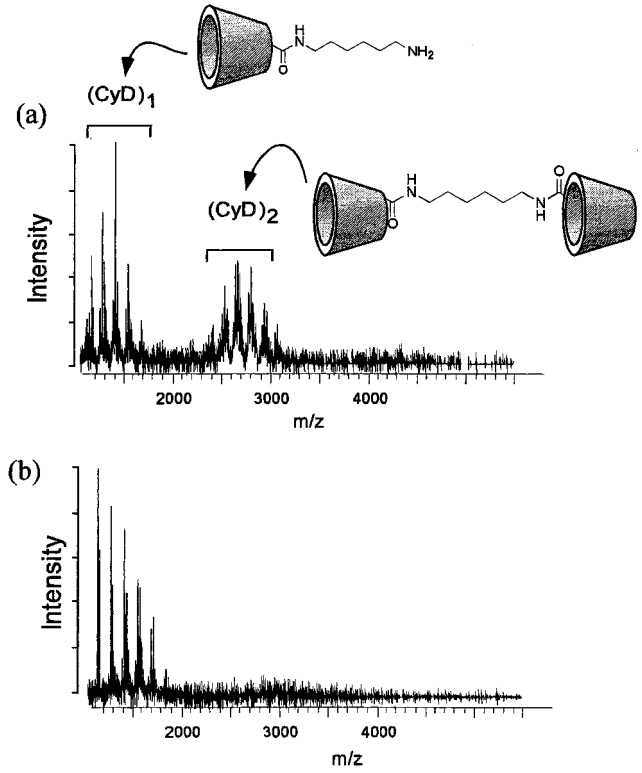

Figure 4. MALDI-TOFMS of HMDI-cross-linked β-CyDs: (a) cholesterol-imprinted and (b) nonimprinted. [β-CyD] = 100 mM, [HMDI] = 200 mM, and [cholesterol] = 50 mM; in dry DMSO at 65 °C for 2 h.

secondary OH groups efficiently react with TDI. This reaction is accelerated by proximity effect induced by the template (vide infra). On the other hand, the reactivity of the primary OHs is not much changed by the imprinting (the two plots in part b are virtually superimposed on each other).

Molecular-Imprinting of DM-β-CyD with Cholesterol. In DM-β-CyD, all the primary OHs and half of the secondary ones are protected by methyl groups, and thus only the secondary OHs (on the C-3 carbons) are available for cross-linking. Yet, the molecular imprinting with cholesterol by using TDI has been successful, and the cholesterol-binding activities are greatly

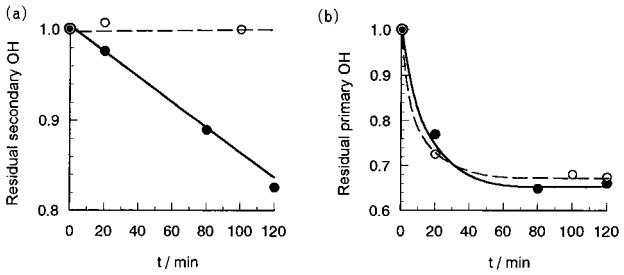

Figure 5. Time courses for the residual amounts of (a) the secondary OH and (b) the primary OH of β-CyD in the reaction with TDI in DMSO-d_6 at 65 °C: (●) in the presence of cholesterol and (○) in its absence. [β-CyD] = 100 mM, [TDI] = 300 mM, and [cholesterol] = 50 mM. The data for the secondary OH in the late stage of the reaction under the imprinting conditions could not be obtained, since the signals were broadened and could not be separated from the signals for H^1 of β-CyD.

improved (see Figure 2 in the Supporting Information). Significant roles of the secondary OHs in the molecular-imprinting of β-CyD are strongly indicated. These arguments are concretely supported by the MALDI-TOFMS study. As shown in Figure 6, dimers of DM-β-CyD (M = 2836−3428) are efficiently formed in the presence of cholesterol (a), whereas they are marginal in its absence (b).

Discussion

Origins of the Molecular-Imprinting Effects. The MALDI-TOFMS has clearly shown that the molecular-imprinting of β-CyD by cholesterol and stigmasterol[14] is attributable to the promoted formation of dimers and trimers of β-CyD (Figures 2a and 4a). These steroids are too large to be accommodated in the cavity of one β-CyD molecule. In these ordered assemblies of β-CyD, however, two (or three) β-CyDs are regularly placed, and cooperatively bind these large guests. The structures of templates are memorized in terms of the orientation of β-CyD molecules. Without the molecular-imprinting, these ordered assemblies cannot be easily formed due to unfavorable entropy change (Figures 2b and 4b). Accordingly, nonimprinted β-CyD polymers show only marginal guest-binding. Inefficiency of progesterone and testosterone as templates is ascribed to the same reason (Figure 2c).

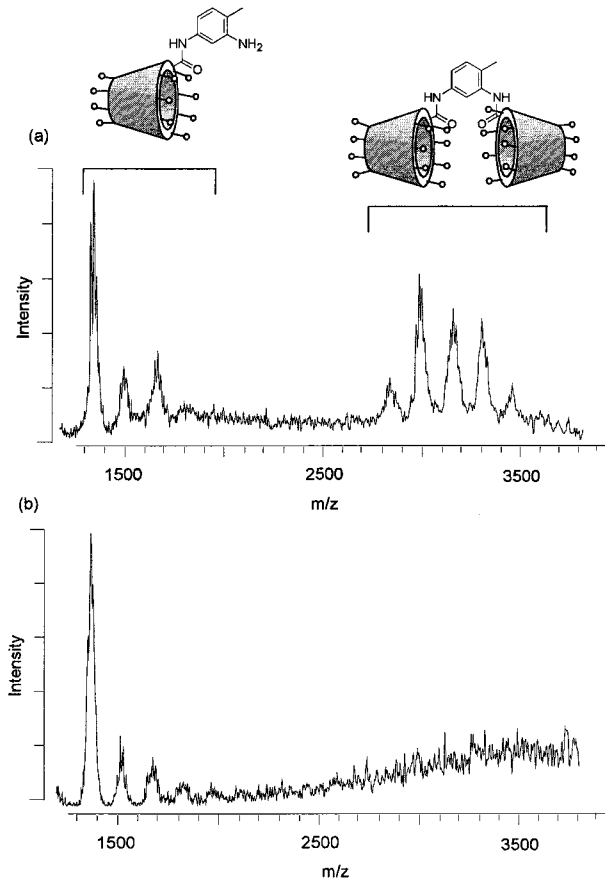

Figure 6. MALDI-TOFMS of TDI-cross-linked DM-β-CyDs: (a) cholesterol-imprinted and (b) nonimprinted. [DM-β-CyD] = 100 mM, [TDI] = 300 mM, and [cholesterol] = 50 mM; in dry DMSO at 65 °C for 2 h.

Previously,[14] the structures of guest-binding sites of cholesterol-imprinted β-CyD polymers were investigated by using structural probes. Binding-activities were appreciable only when a guest was larger than 10−11 Å and could simultaneously interact with both of the β-CyD groups in the β-CyD dimer.[31] Independently, dimers of β-CyD were prepared by Breslow[10] and Reinhoudt[11] with elegant synthetic procedures, showing efficient steroid-binding. On the basis of these results, it was proposed that the β-CyD dimer is the guest-binding site. The present spectroscopic analysis has provided direct evidence for this proposal.

Mechanism of the Molecular-Imprinting. The molecular-imprinting reactions of β-CyD are characterized by the following two factors: (1) the bridging of two β-CyDs by the cross-linking agent is enormously accelerated by the template and (2) the secondary OH groups of β-CyD, which are otherwise poor in reactivity, are notably participating in the reactions. Moreover, cholesterol and β-CyD form a 1:1 adduct.[13b] Nevertheless, the dimerization and trimerization of β-CyDs occur efficiently in the presence of the template. From these results, the mechanism of the present imprinting is proposed as depicted in Figure 7. First, one of the two isocyanate groups of cross-linking agent reacts with β-CyD and $\mathbf{1^{(1)}}$ is produced. This reaction mainly occurs (either with the template or without it) at the primary OH groups, which are more reactive. When the other isocyanate group reacts with another β-CyD under the imprinting conditions, however, the reaction preferentially takes place at the secondary OH. According to the kinetic study in Figure 5a, more than 10% of the secondary OHs of β-CyD have been consumed in 80 min. This indicates that at least one secondary OH of all the β-CyDs has reacted with an isocyanate (note that β-CyD has 14 secondary OH groups, and they are inactive in the absence of the template).[32] Most of the β-CyD dimers are formed by bridging the primary OH of one β-CyD with the secondary OH of another β-CyD. Here, the β-CyD bearing the isocyanate forms an inclusion complex with cholesterol, which in turn interacts with another β-CyD. Since cholesterol is too long to be accommodated in one β-CyD cavity, the second β-CyD dynamically accesses to this 1:1 complex. Importantly, the cholesterol penetrates into the cavity of the second β-CyD from its secondary hydroxyl side, rather than from the primary hydroxyl side (this side is more widely open, and most of the CyD inclusion complexes take this orientation).[6a,b] As a result, the secondary OH groups of the second β-CyD are placed near the isocyanate, giving rise to the prompt reaction.[33] As the molecular-imprinting proceeds further, the β-CyD residues in these dimers and trimers are cross-linked by other diisocyanate molecules and frozen in the imprinted polymers. Consistently, a pair of β-CyDs in the trimers $\mathbf{3^{(n)}}$ are doubly bridged (see Figure 3).[26]

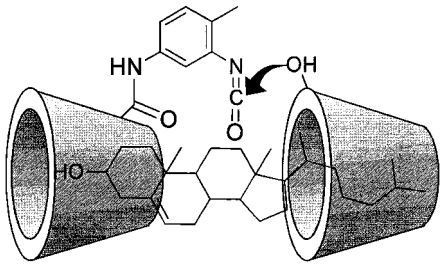

Figure 7. Proposed mechanism for the molecular-imprinting of β-CyD with cholesterol (cross-linking agent: TDI).

All these arguments are in accord with the fact that progesterone and testosterone, which have no long hydrophobic chains, are inactive as the templates and hardly promote the formation of the β-CyD dimers. In the molecular-imprinting of DM-β-CyD, the secondary OH residues are connected by cross-linking agent, since no primary OHs are available.[34] Assumedly, two DM-β-CyD molecules are interacting with one cholesterol, and the secondary OH sides of them are placed nearby. The bridging between them can be promoted by the proximity effect.

Conclusion

The present spectroscopy has shown that ordered assemblies of β-CyD are efficiently formed during the molecular-imprinting processes. Dynamic β-CyD-template complexes are immobilized in polymer networks.[35] The templates govern the mutual orientation of β-CyD molecules, and further dictate the sites

(31) Note that this cholesterol-imprinted polymer selectively binds cholesterol and thus the activities toward these structural probes are far smaller than its cholesterol-binding activity.

(32) The conversion should be 7.1% (1/14), when one secondary OH of all the β-CyD is reacted.

(33) These dynamic processes do not need a stable 2:1 complex.

(34) The secondary OH groups of DM-β-CyD can be more reactive than that of the parent β-CyD, because of the more flexible structure of DM-β-CyD. The intramolecular hydrogen-bonding network of the secondary OH groups, which makes the cyclic structure of β-CyD rigid, is absent in this derivative (see ref 6b).

(35) (a) Huc, I.; Lehn, J.-M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2106. (b) Eliseev, A. V.; Nelen, M. I. *J. Am. Chem. Soc.* **1997**, *119*, 1147. (c) Cousins, G. R. L.; Poulsen, S.-A.; Sanders, J. K. M. *Curr. Opin. Chem. Biol.* **2000**, *4*, 270 and references therein.

where the bridging reactions occur (the secondary hydroxyl groups, which are otherwise inactive, dominantly participate in these reactions). These β-CyD assemblies are the guest-binding sites. They are so abundant in the imprinted polymers that both the binding-capacity and binding-selectivity are improved. The mechanism of molecular imprinting has been made evident. By use of similar methodology, artificial receptors for versatile nanometer-scaled guests can be obtained without laborious synthetic procedures. Although molecular-imprinting methods have been widely used, little information has been available on the mechanistic details. This study sheds some light on these matters.

Experimental Section

Materials. Steroids, β-CyD, and DM-β-CyD were purchased from Tokyo Kasei Co. Ltd. and were dried in vacuo at 70 °C for 24 h before use. DMSO was dried with molecular sieve 4A and then distilled under reduced pressure. Water was purified by a Millipore Milli-XQ purification system.

MALDI-TOFMS Analyses. Water-soluble specimens were prepared by incubating β-CyD (100 mM) with TDI or HMDI (200 mM) in the presence of the template molecule (50 mM) in dry DMSO at 65 °C for 2 h. The mole ratio of the cross-linking agent to β-CyD was 2.0, which was smaller than the value (6.4) employed previously for the preparation of water-insoluble polymeric receptors.[13−15] All the reactions proceeded in homogeneous solutions. Subsequently, the mixtures were poured into acetone, and the resultant powder was carefully washed to remove the template, DMSO, and the cross-linking agent. Nonimprinting reactions were achieved in a similar way, except for the absence of template.

The MALDI-TOFMS spectra were measured in the positive mode with a Shimadzu/KRATOS KOMPACT TYPE I spectrometer. Imprinted or nonimprinted specimens obtained above (1 mg) were dissolved in 1 mL of water, and a 1 μL portion was mixed with 1 μL of a 1:9 (v/v) water/ethanol mixture containing 10 mM 2,4-dihydroxybenzoic acid (matrix). Laser wavelength and its power were 337 nm and 110 mV, respectively. Mass values were calibrated with insulin (M = 5734) and 3,5-dimethoxy-4-hydroxycinnamic acid (M = 206) as standards. No covalent and noncovalent adducts between β-CyD (or DM-β-CyD) and cholesterol (or other steroids) were observed.

NMR Measurements. In the presence of cholesterol, β-CyD (100 mM) was reacted with TDI (300 mM) in dry DMSO-d_6 at 65 °C. At appropriate reaction times, ^{1}H NMR spectra were measured on a JEOL α-500 (500 MHz) spectrometer. The signals were assigned by using a one-dimensional ^{1}H spectrum and a ^{1}H−^{1}H COSY spectrum.

Preparation of Imprinted DM-β-CyD Polymers and Assay of Their Binding Activities. Water-soluble specimens for the mass spectroscopy were obtained by reacting DM-β-CyD (100 mM) with TDI (300 mM) in dry DMSO at 65 °C for 2 h in the presence of a template (50 mM). After DMSO was evaporated, the polymer was washed with acetone, THF, and hot water, and then dried in vacuo at 70 °C for 24 h. In the preparation of water-insoluble polymers, the mole ratio of TDI to DM-β-CyD was increased to 6.4. The binding activities of these polymers were measured as reported before.[14]

Filtration of the Imprinted Mixtures. Water-soluble products of cholesterol-imprinted reactions (1 mg), obtained as described above for MALDI-TOFMS, were dissolved in 100 μL of water. Then, the solution was poured onto a cellulose membrane filter (Millipore Ultrafree-MC) and centrifuged at 12 000 rpm. The remainder left on the top of the filter was washed with 300 μL of water. The specimen was dissolved in 10 μL of water and analyzed by MALDI-TOFMS.

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Supporting Information Available: Figures showing the time curses of the peak intensity of dimeric CyD, cholesterol-binding activities of the cholesterol-imprinted and non-imprintes DM-β-CyD polymers, and MALDI-TOFMS (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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