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Skeleton-selective fluorescent chemosensor based on cyclodextrin bearing a 4-amino-7-nitrobenz-2-oxa-1,3-diazole moiety

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A new type of fluorescent chemosensor, based on modified cyclodextrins bearing the fluorophore unit NBD–amine, was prepared. One of these new chemosensors, **NC0βCD**, is sensitive to adamantane and borneol derivatives, which have a comparatively spherical shape that fits the β-CD cavity, but is not sensitive to bile acids, which are strongly bound by the native β-CD. Even in the presence of a bile acid, **NC0βCD** can detect 1-adamantanol. Another of this new type of chemosensors, **NC0γCD**, is sensitive to bile acids but not to adamantane derivatives. The response of the new type of chemosensors to a guest was an increase in the fluorescence intensity.

Introduction

The signal transduction of molecule- or metal-binding events into spectroscopic changes has recently emerged as an important application of supramolecular chemistry.[1–4] We have prepared many kinds of chromophore-modified cyclodextrins (CDs) as chemosensors for molecule recognition.[5–12] Colorless neutral molecules can be detected as changes in the intensity of fluorescence, absorption or circular dichroism using chemosensors based on chromophore-modified CDs. The mechanism of these chemosensors is shown in Fig. 1. The chromophore-modified CDs can adopt some conformations in aqueous solution. The conformation equilibrium can be explained by the simplified two-state model shown in Fig. 1.[9–10] The 'self-inclusion state' is usually the major conformation. An 'induced-fit' conformational change of the chromophore-modified CD occurs in association with accommodation of the guest, which displaces the chromophore from the inside to the outside of the CD cavity. The 'non-self-inclusion state' increases with an increase in the guest concentration. In the case of a fluorophore, the fluorescent CD exhibits a strong fluorescence in the self-inclusion state due to the hydrophobic environment of the CD cavity, and exclusion of the fluorophore from the cavity to the bulk water weakens its fluorescence intensity. The extent of the variation in fluorescence intensity depends on the affinity of the chemosensor for a guest. This chemosensor system is effective for detecting molecules, but it has some defects. (1) Self-inclusion of the chromophore can inhibit accommodation of the guest. For example, the self-inclusion state of dansyl-D-leucine-modified β-CD is twice as stable as that of dansyl-L-leucine-modified β-CD, and the binding ability of the former is about half that of the latter.[10] (2) Changing the chromophore or spacer unit can alter the selectivity of the chemosensor, but the effect is not large, because the guest selectivity of the chemosensor mainly depends on the selectivity of the CD itself. The affinities of both β-CD and γ-CD for bile acid derivatives are greater than their affinities for adamantane derivatives. Therefore, adamantanol cannot be detected in a mixed solution of a bile acid and adamantanol using conventional chemosensors. (3) For most conventional chemosensors, the detection of a guest is accompanied by a decrease in the fluorescence intensity, although an increase in the emission intensity caused by guest response is more effective for chemical sensing systems. Therefore, we now propose a new method to overcome these defects as shown in Fig. 2. If the chromophore is connected to the CD without an alkyl spacer in the linker, the chromophore cannot be self-included and will remain at the entrance of the CD cavity. In this situation, some water is accommodated in the cavity and the chromophore is surrounded by a hydrophilic environment. If a hydrophobic guest then enters the cavity such that the hydrophobic face of the guest interacts with the chromophore, the chromophore will be located in a more hydrophobic environment. When the chromophore is a fluorophore, its fluorescence intensity will be weak in the absence of guests and stronger in the presence of hydrophobic guests. This mechanism is expected to produce a new selectivity in the chemosensor, because a more limited variety of guests will have the correct shape capable of increasing fluorescence intensity. Furthermore, the fluorophore will act as a hydrophobic cap to increase the affinity of the chemosensor to guests. We here report a new type of chemosensor that can detect adamantanol even in the presence of a bile acid, even though the affinity of the native β-CD is greater for bile acids than for adamantanol.

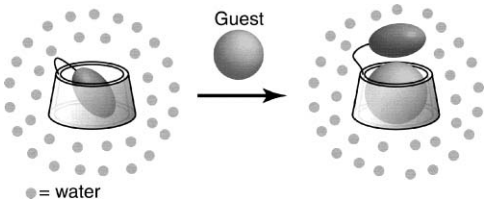

Fig. 1 Guest-induced conformational change of a conventional chromophore-modified cyclodextrin.

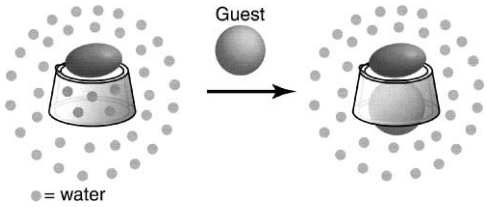

Fig. 2 Guest-induced conformational change of a new type of chromophore-modified cyclodextrin.

Results and discussion

Syntheses of NBD–amine-modified CDs

We selected 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD–amine) as a fluorophore for our new type of chemosensor (Chart 1). NBD–Cl is a fluorogenic reagent that is not fluorescent itself but reacts with an amine group to form the fluorescent

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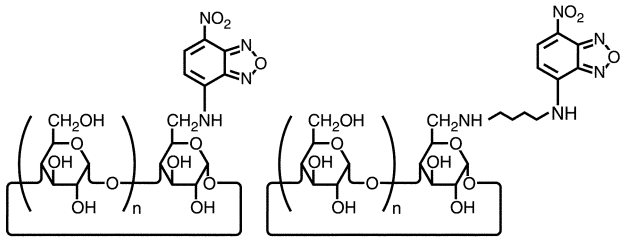

Chart 1 Structures of NBD–amine-modified cyclodextrins.

derivative, NBD–amine.[13–15] NBD–amine displays the interesting property of fluorescing weakly in water and strongly in organic solvents, membranes or hydrophobic environments. The new type of chemosensor **NC0βCD** was synthesized by the reaction of mono-6-amino-6-deoxy-β-CD with 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD–Cl). The fluorophore unit (NBD–amine) is directly connected to the CD framework of **NC0βCD**. A reference compound, **NC4βCD**, which has a butylenediamine spacer, was synthesized from 6-(4-aminobutylamino)-6-deoxy-β-CD. The NBD unit of **NC4βCD** would be self-included and the guest selectivity of **NC4βCD** would be similar to that of the conventional CD-based chemosensors. The chemosensors **NC0γCD** and **NC4γCD**, which have a larger cavity, were also prepared.

Absorption and fluorescence spectra of NC0βCD and NC4βCD

The absorption spectra of **NC0βCD** and **NC4βCD** in the presence of various amounts of 1-adamantanol (1-AdOH) were measured in phosphate buffer (pH 7.0) as shown in Fig. 3. The peak intensity of **NC0βCD** was decreased, while that of **NC4βCD** was increased, by increasing the concentration of 1-AdOH. This result indicates that the hydrophobic guest increases the hydrophobicity near the NBD–amine moiety of **NC0βCD**, whereas the guest displaces the NBD–amine moiety of **NC4βCD** from the hydrophobic CD cavity to the bulk water, because the molar absorption coefficient (ε) of NBD–amine derivatives rise with increasing solvent polarity.[14] We observed

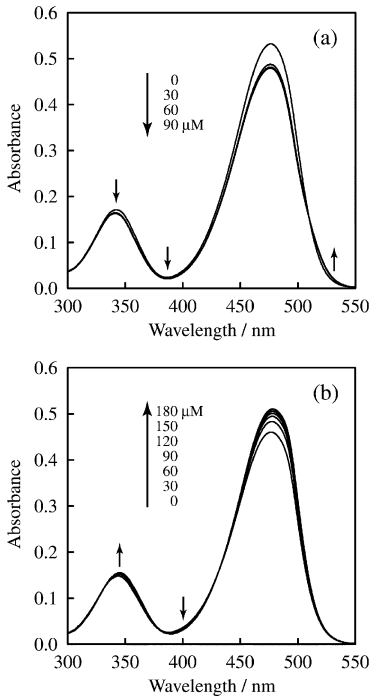

Fig. 3 Absorption spectra of (a) **NC0βCD** (2×10^{-5} M) and (b) **NC4βCD** (2×10^{-5} M) in the presence of various concentrations of 1-AdOH in phosphate buffer (200 mM, pH 7.0) at 25 °C.

the isosbestic point for each host (510 nm for **NC0βCD**, 434 nm for **NC4βCD**), and the wavelength at the isosbestic point was chosen as the excitation wavelength for fluorescence measurements. The fluorescence spectra of **NC0βCD** and **NC4βCD** in phosphate buffer (pH 7.0) have emission bands with peaks at 569.5 and 539.5 nm, respectively, as shown in Fig. 4. This difference in the emission maximum wavelength results from a difference in hydrophobicity near each NBD–amine moiety. It means that the NBD–amine moiety of **NC4βCD**, which has the butylenediamine spacer, penetrates more deeply into the hydrophobic CD cavity than that of **NC0βCD**, which has no spacer. The fluorescence intensity of **NC0βCD** rose upon the addition of 1-AdOH, indicating an increase in hydrophobicity near the NBD–amine moiety induced by accommodation of the guest (Fig. 4a). By contrast, the fluorescence intensity of **NC4βCD** decreased upon the addition of 1-AdOH, indicating a positional change of the fluorophore from the inside to the outside of the CD cavity (Fig. 4b). The peak maxima of the fluorescence spectra of **NC0βCD** and **NC4βCD** shifted to shorter and longer wavelength with increasing the guest concentration, respectively. This also indicates that the hydrophobicity around the NBD unit changes upon the addition of the guest.

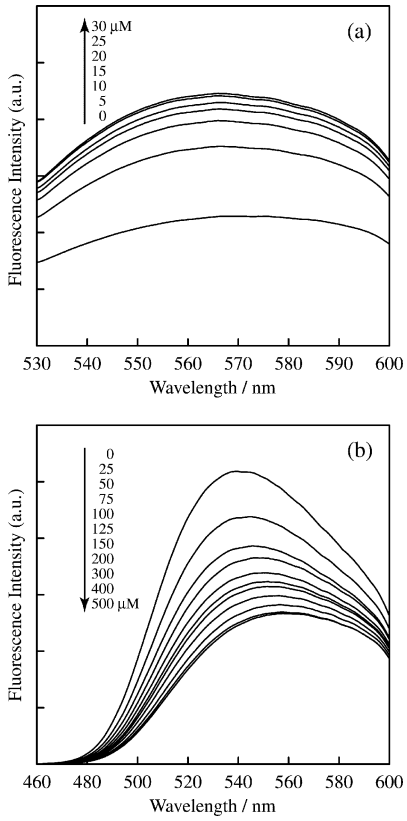

Fig. 4 (a) Fluorescence spectra of **NC0βCD** (5×10^{-6} M) in the presence of various concentrations of 1-AdOH in phosphate buffer (200 mM, pH 7.0) at 25 °C; the excitation wavelength was 510 nm. (b) Fluorescence spectra of **NC4βCD** (5×10^{-6} M) in the same condition as **NC0βCD**; the excitation wavelength was 434 nm.

Guest selectivity of the chemosensors

The guest selectivity of the chemosensor was evaluated by the sensitivity parameter ($\Delta I/I_0$; $\Delta I = I - I_0$ where I_0 and I denote the fluorescence intensities in the absence and presence of a guest, respectively). The structures of the guests for evaluation of the guest selectivities of the chemosensors are shown in Chart 2. The native β-CD strongly accommodates the bile acid derivatives and moderately accommodates the adamantane and borneol derivatives. The affinity of the native β-CD for small molecules is weak.

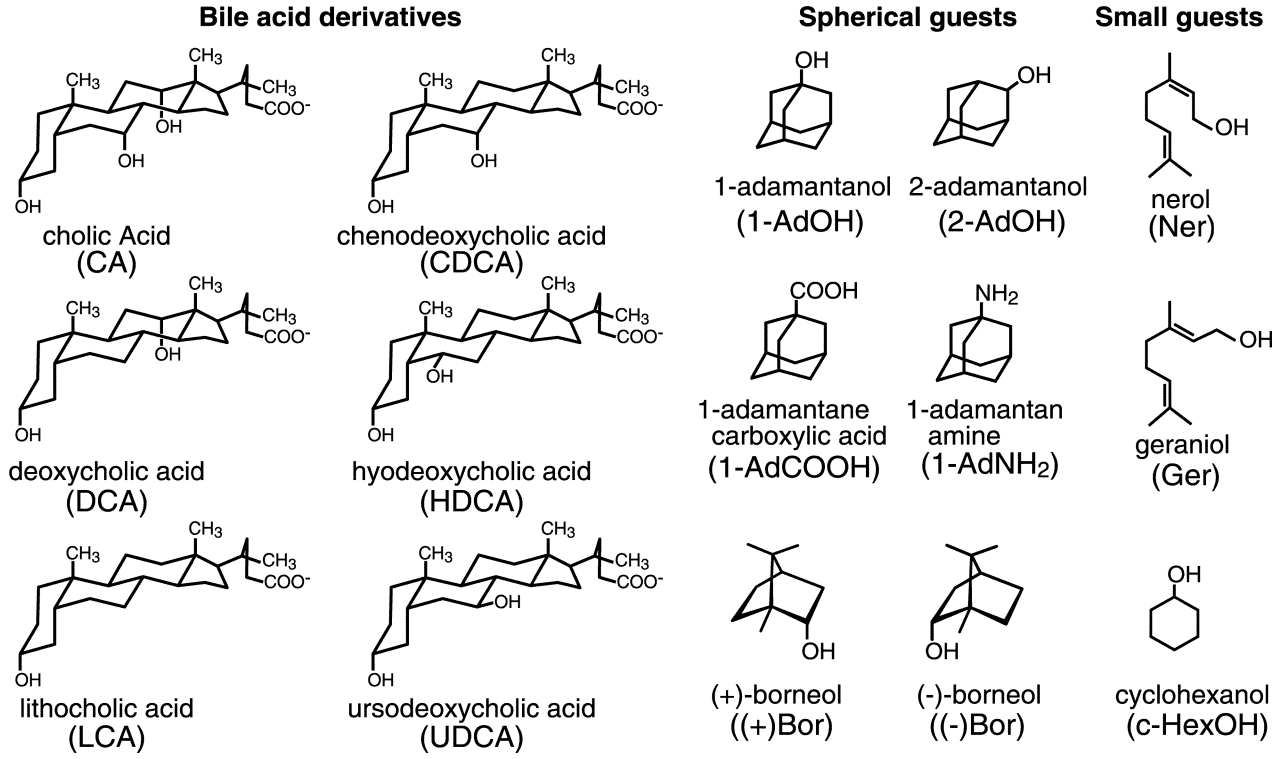

Chart 2 Structures of guests.

Fig. 5 shows the guest selectivity pattern of **NC4βCD** (5×10^{-6} M) for various guests each at a guest concentration of 1×10^{-5} M. The sensitivity of **NC4βCD** to the guests decreases in the following order: bile acids > adamantane derivatives > borneol derivatives > small molecules. This selectivity is characteristic of conventional CD-based chemosensors.[4–12] **NC4βCD** is less sensitive to CA and DCA than other bile acid derivatives. This pattern of selectivity is similar to that of conventional CD-based chemosensors.

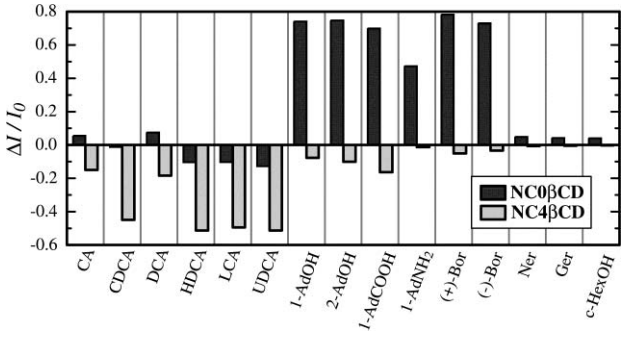

Fig. 5 Sensitivity parameters ($\Delta I/I_0$) of **NC0βCD** (5×10^{-6} M) and **NC4βCD** (5×10^{-6} M) for various guests (each at 1×10^{-5} M) in phosphate buffer (200 mM, pH 7.0) at 25 °C. The excitation wavelength was 510 nm for **NC0βCD** and 434 nm for **NC4βCD**. The emission wavelength was 569.5 nm for **NC0βCD** and 539.5 nm for **NC4βCD**.

By contrast, the new type of chemosensor **NC0βCD** is much more sensitive to the adamantane and borneol derivatives. The shape of these guests is comparatively spherical and their size matches the β-CD cavity more closely. The response of **NC0βCD** to these guests is a large increase in the fluorescence intensity; by contrast, the fluorescence intensity of **NC4βCD** is decreased by the addition of guests. It is noteworthy that **NC0βCD** is not sensitive to bile acids, although bile acids are strongly bound by the native β-CD.[16–17]

Maximum variation and binding constants of the chemosensors for guests

The plot of $\Delta I/I_0$ *versus* the guest concentration can be fitted by an equation for the 1 : 1 host–guest complex.[9] The binding constant (K_b) and $\Delta I_{max}/I_0$ can be obtained from this curve fitting analysis.[9] The $\Delta I_{max}/I_0$ values of **NC0βCD** for CDCA, HDCA, LCA, and UDCA are too small to calculate the binding constants; therefore, the binding constants for these derivatives were obtained by another method as described below. ΔI_{max} is the fluorescence spectra variation for the addition of an infinite quantity of the guest and the $\Delta I_{max}/I_0$ value provides information about the environment around the fluorophore in the complex. The $\Delta I_{max}/I_0$ values of **NC0βCD** and **NC4βCD** are shown in Table 1. The $\Delta I_{max}/I_0$ of **NC4βCD** is a similar negative value for each guest. By contrast, the $\Delta I_{max}/I_0$ of **NC0βCD** for each guest differs considerably. For example, the $\Delta I_{max}/I_0$ values for bile acids are small negative or positive values, whereas those for the adamantane and borneol derivatives are large positive values. This observation suggests that the environment around the fluorophore in the **NC4βCD**–guest complex is almost the same for each guest, whereas that in the **NC0βCD**–guest complex is different for each guest. This difference in the fluorophore environment causes the characteristic sensing patterns.

The binding constants for adamantane derivatives of **NC0βCD** are larger than those of **NC4βCD** (Table 2). This finding suggests that the NBD unit also acts as a hydrophobic cap for guest binding rather than a binding inhibitor.

Competition assay for UDCA

A competition experiment was performed to confirm whether **NC0βCD** could accommodate a bile acid or not. We reasoned that if UDCA can be strongly accommodated by **NC0βCD**, similar to the native β-CD, then 1-AdOH in the **NC0βCD**–1-AdOH complex would be replaced by UDCA. Furthermore, because the **NC0βCD**–1-AdOH complex shows a strong emission but the **NC0βCD**–UDCA complex shows a weak fluorescence, the addition of UDCA to the solution of the **NC0βCD**–1-AdOH complex should decrease the fluorescence intensity. The

Table 1 $\Delta I_{max}/I_0$ of **NC0βCD**, **NC4βCD**, and **NC0γCD**

Guest	$\Delta I_{max}/I_0$		
	NC0βCD	**NC4βCD**	**NC0γCD**
CA	0.16	−0.60	1.59
CDCA	−0.14[b]	−0.61	1.01
DCA	0.65	−0.57	1.07
HDCA	−0.13[b]	−0.57	0.67
LCA	−0.15[b]	−0.59	0.54
UDCA	−0.15[b]	−0.57	0.58
1-AdOH	1.02	−0.60	0.37
2-AdOH	0.96	−0.58	0.37
1-AdCOOH	0.89	−0.64	0.61
1-AdNH$_2$	1.36	−0.65	[a]
(+)-Bor	1.30	−0.52	0.45
(−)-Bor	1.36	−0.57	0.44
Ner	0.95	−0.47	0.44
Ger	0.83	−0.60	0.31
c-HexOH	1.00	−0.53	[a]

[a] The accurate value could not determined due to small changes in fluorescence. [b] The competition assay was used to obtain the value.

Table 2 Binding constants (K_b) of **NC0βCD**, **NC4βCD**, and **NC0γCD**

Guest	$K_b/10^4$ M^{-1}		
	NC0βCD	**NC4βCD**	**NC0γCD**
CA	6.7	3.8	4.1
CDCA	5.2[b]	43	15
DCA	1.4	5.6	22
HDCA	81[b]	150	31
LCA	110[b]	90	370
UDCA	60[b]	160	15
1-AdOH	42	1.6	0.15
2-AdOH	58	2.3	0.16
1-AdCOOH	59	3.9	0.080
1-AdNH$_2$	6.4	0.2	[a]
(+)-Bor	21	1.1	0.72
(−)-Bor	16	0.65	0.65
Ner	0.56	0.13	0.22
Ger	0.55	0.071	0.11
c-HexOH	0.43	0.042	[a]

[a] The accurate value could not determined due to small changes in fluorescence. [b] The competition assay was used to obtain the value.

fluorescence intensity did indeed decrease with the addition of UDCA as shown in Fig. 6. This variation was fitted by an equation for a competition assay.[18] The binding constant (K_b) and $\Delta I_{max}/I_0$ of **NC0βCD** for the bile acid derivatives can be obtained from this curve fitting analysis as the usual method cannot be used to calculate the binding constants. The binding

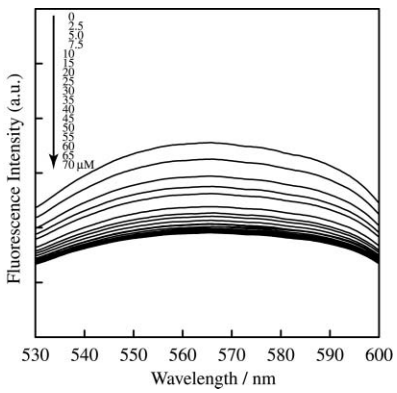

Fig. 6 Fluorescence spectra of **NC0βCD** (5×10^{-6} M) with 1-AdOH (1×10^{-5} M) in the absence and presence of various concentrations of UDCA in phosphate buffer (200 mM, pH 7.0) at 25 °C; the excitation wavelength was 510 nm.

constant of **NC0βCD** for UDCA is larger than that for 1-AdOH but $\Delta I_{max}/I_0$ for UDCA is a small negative value. This result indicates that UDCA is accommodated in **NC0βCD**, but it does not change the fluorescence intensity of the NBD–amine.

^{1}H NMR of the complex of NC0βCD with 1-AdOH and UDCA

The interaction between the NBD moiety and a guest was investigated by ^{1}H NMR. With the addition of 1-AdOH to a solution of **NC0βCD** in D_2O, the resonances for the NBD moiety broadened as shown in Fig. 7. This change suggests that the motion of NBD was restricted by the van der Waals interactions with 1-AdOH. This observation indicates that the NBD moiety is still positioned at the entrance of the CD cavity, even when 1-AdOH is accommodated. By contrast, with the addition of UDCA to a solution of **NC0βCD** in D_2O, the resonances for the NBD moiety sharpened. Therefore, the NBD moiety must move away from the entrance of the CD cavity and the motion of the NBD moiety is not restricted. These different behaviors of the NBD moiety cause the difference in the fluorescence variation observed upon addition of the guest. The resonances for the NBD moiety of **NC0βCD** alone [Fig. 7(a)] are broader than those of **NC0βCD** in the presence of UDCA [Fig. 7(c)]. This suggests that the motion of the NBD moiety of **NC0βCD** is restricted in the absence of the guest, because the NBD moiety interacts with the rim of the CD cavity.

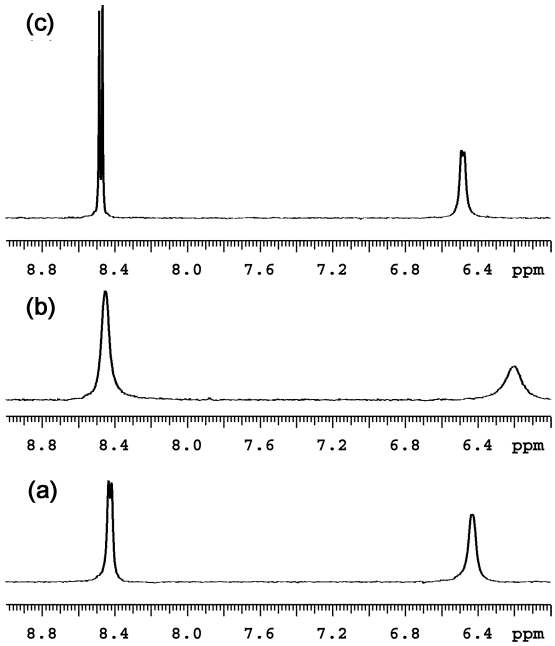

Fig. 7 ^{1}H NMR spectra of **NC0βCD** (a) alone and in the presence of (b) 1-AdOH or (c) UDCA showing the region of NBD protons; [**NC0βCD**] = [1-AdOH] = [UDCA] = 5×10^{-4} M.

Assay of a guest mixture by NC0βCD

We tried to detect 1-AdOH in the presence of a bile acid by using **NC0βCD** as a chemosensor. An assay for a guest mixture using a chemosensor based on the CD derivatives has not previously been reported. The conventional fluorophore-modified CD cannot monitor the adamantane derivatives in the presence of bile acids, because its affinity is greater for bile acids than for adamantane derivatives. The relative sensitivity parameters $((\Delta I/I_0)_{mix}/(\Delta I/I_0)_{1\text{-AdOH}})$ of **NC0βCD** (5×10^{-6} M) were evaluated for a solution containing both bile acid (1×10^{-5} M) and 1-AdOH (1×10^{-5} M). In Fig. 8, the sensitivity parameters have been normalized to the sensitivity parameters for the addition of 1-AdOH alone. The relative sensitivity parameters of **NC0βCD** for each mixture are positive and are comparable to that for the addition of 1-AdOH alone. In the case

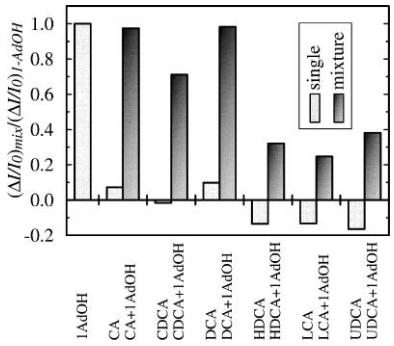

Fig. 8 Relative sensitivity parameters $((\Delta I/I_0)_{mix}/(\Delta I/I_0)_{1\text{-AdOH}})$ of **NC0βCD** (5×10^{-6} M) for mixtures of guests (each at 1×10^{-5} M) in phosphate buffer (200 mM, pH 7.0) at 25 °C; the excitation wavelength was 510 nm. The emission wavelength was 569.5 nm.

of HDCA, LCA, and UDCA, the relative sensitivity parameters for the mixtures are not high, but the chemosensor response is an increase in the fluorescence intensity. Because the responses to these bile acids on their own are a small decrease in intensity, we can detect adamantanol even in the mixtures containing these derivatives. To our knowledge, this is the first time that adamantanol has been detected in the presence of a bile acid by a CD-based chemosensor.

Sensitivity parameters of NC0γCD and NC4γCD

NC0βCD shows a relatively weak response to bile acids, because there is not enough space between the rim of the CD and the NBD unit to incorporate the alkyl chain at the D ring of a bile acid, and the NBD unit is displaced by accommodation of the guest. Because γ-CD has enough space for the alkyl chain of a bile acid and the γ-CD cavity is too large for the adamantane derivatives, we expected to produce a chemosensor that is sensitive to bile acid derivatives but has no response to adamantane derivatives using the γ-CD derivative. The sensitivity parameters of **NC0γCD** and **NC4γCD** for various guests are shown in Fig. 9. **NC0γCD** is relatively sensitive to each bile acid but has no response to the adamantane derivatives, and **NC4γCD** is not sensitive to any of the guests. The responses of **NC0γCD** to the guests are also increases in the fluorescence intensity. The guest–response pattern of **NC0γCD** differs from that of **NC0βCD**. Take together, these results indicate that we can now easily discriminate between bile acids and adamantane derivatives at any concentration by the combined use of **NC0βCD** and **NC0γCD**.

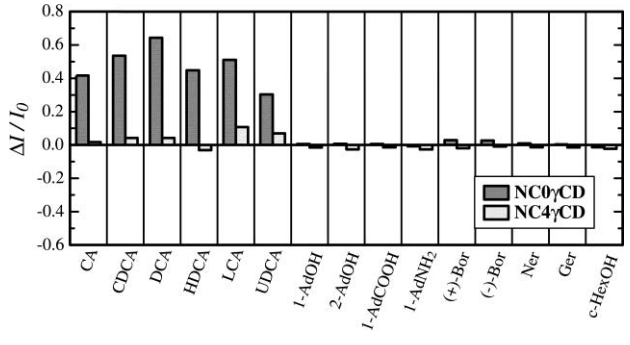

Fig. 9 Sensitivity parameters ($\Delta I/I_0$) of **NC0γCD** (5×10^{-6} M) and **NC4γCD** (5×10^{-6} M) for various guests (each at 1×10^{-5} M) in phosphate buffer (200 mM, pH 7.0) at 25 °C; the excitation wavelength was 500 nm for **NC0γCD** and 431 nm for **NC4γCD**. The emission wavelength was 566 nm for **NC0γCD** and 556 nm for **NC4γCD**.

Conclusions

We have prepared two new kinds of chemosensor: one is sensitive to adamantane derivatives but not to bile acid derivatives; the other is sensitive to bile acid derivatives but not to adamantane derivatives. We can detect both adamantane derivatives and bile acid derivatives in a mixture by the combined use of these new chemosensors. We expect to be able to develop a pattern-based assay by the combined use of these types of chemosensor.

Experimental

Materials

CDs were kindly donated by Nihon Shokuhin Kako Co., Ltd, and were used without further purification. Reagents were purchased from Sigma-Aldrich Co., Tokyo Kasei Kogyo Co., Ltd, and Wako Pure Chemical Industries, Ltd, and were used without further purification. Deuterium oxide for NMR measurements was obtained from Merck Co.

Measurements

Reverse phase HPLC was performed using a HITACHI HPLC system comprising a HITACHI L-7100 Intelligent Pump, HITACHI D-7500 Chromato-Integrator and HITACHI L-7400 UV-VIS Detector. ^{1}H NMR spectra were measured on a Varian VXR 500S spectrometer (500 MHz). HDO ($\delta = 4.70$) was used as an internal standard. Matrix assisted laser desorption/ionization and time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a SHIMADZU KRATOS KOMPACT MALDI III mass spectrometer using α-cyano-4-hydroxycinnamic acid as a matrix. Thin-layer chromatography (TLC, *n*-butanol–ethanol–water = 5 : 4 : 3, and conc. NH_3(aq.)–ethyl acetate–2-propanol–water = 1 : 3 : 5 : 4) was carried out with silica gel F254 (Merck Co.). Absorption spectra were measured on a SHIMADZU UV-Visible spectrophotometer UV-2550. Fluorescence spectra were measured on a HITACHI fluorescence spectrophotometer F-2500.

Syntheses

NC0βCD: 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD–Cl, 28.2 mg, 0.140 mmol) was added to a DMF (2.0 ml)–methanol (2.5 ml) solution containing triethylamine (6.1 μl, 0.044 mmol) and mono-6-deoxy-6-amino-β-cyclodextrin[11] (20 mg, 0.0176 mmol). The reaction mixture was stirred at room temperature for 4.5 h. Then, the solution was poured into acetone (140 ml), and the precipitates were dried *in vacuo* overnight, giving 18.6 mg of crude product. This crude product was purified by reverse phase HPLC, and the final product was obtained as a yellow powder (12.6 mg, 55.2% yield). ^{1}H NMR (D_2O, 500 MHz): δ 4.89–5.13 (m, 7H, H-1), 6.43 (bs, 1H, aromatic), 8.45 (d, 1H, aromatic). MALDI-TOF MS: m/z 1320.9 (calcd for $[M + Na]^+$, 1320.1).

NC4βCD was synthesized by the reaction of mono-6-deoxy-6-(4-aminobutylamino)-β-cyclodextrin[12] with NBD–Cl in a DMF–methanol solution containing triethylamine in the same manner as described for **NC0βCD**. The final product was obtained as a yellow powder (18.5 mg, 40.8% yield). ^{1}H NMR (D_2O, 500 MHz): δ 1.77–1.87 (m, 4H, methylene), 4.97–5.04 (m, 7H, H-1), 6.38 (d, 1H, aromatic), 8.61 (d, 1H, aromatic). MALDI-TOF MS: m/z 1390.9 (calcd for $[M + Na]^+$, 1391.2).

NC0γCD was synthesized by the reaction of mono-6-deoxy-6-amino-γ-cyclodextrin[11] with NBD–Cl in the same manner as described for **NC0βCD** (8.0 mg, 9.6% yield). ^{1}H NMR (D_2O, 500 MHz): δ 4.93–5.22 (m, 8H, H-1), 6.36 (d, 1H, aromatic), 8.42 (d, 1H, aromatic). MALDI-TOF MS: m/z 1482.1 (calcd for $[M + Na]^+$, 1482.2).

NC4γCD was synthesized by the reaction of mono-6-deoxy-6-(4-aminobutylamino)-γ-cyclodextrin[12] with NBD–Cl in the same manner as described for **NC0βCD** (6.1 mg, 13.6% yield). ^{1}H NMR (D_2O, 500 MHz): δ 1.84–1.95 (m, 4H, methylene), 4.97–5.08 (m, 8H, H-1), 6.22 (d, 1H, aromatic), 8.19 (d, 1H, aromatic). MALDI-TOF MS: m/z 1552.7 (calcd for $[M + Na]^+$, 1553.4).

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