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Direct assay for α-amylase using fluorophore-modified cyclodextrins

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Abstract—A simple assay method for α-amylase was developed based on fluorophore-modified cyclodextrins (CDs). Four kinds of CD derivatives bearing a 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD-amine) moiety were prepared as artificial substrates for the assay method. The fluorescence intensity of all the NBD-amine-modified CDs decreased upon addition of *Aspergillus oryzae* α-amylase, indicating a reduction in hydrophobicity near the NBD-amine moiety induced by hydrolysis of the CD ring. **NC4γCD**, having a γ-CD and an amino-tetramethylene spacer, was the most sensitive substrate for the α-amylase assay. The initial rate of hydrolysis of **NC4γCD** displayed a liner correlation to the concentration of the α-amylase. **NC4γCD** was sensitive to the α-amylase but was not sensitive to guest compounds that were accommodated by the native CDs.

1. Introduction

α-Amylase (EC 3.2.1.1) is an important *endo*-type carbohydrase that hydrolyzes α-1,4 glycosidic linkages of D-glucose oligomers and polymers. It is a key enzyme of carbohydrate metabolism in mammals, plants, and bacteria. Measurement of α-amylase activity is very important not only in the food industry but also for the diagnosis of pancreatic and salivary diseases. Many assay methods for α-amylase activity have been reported which use simple maltooligosaccharide derivatives bearing a chromophore or fluorophore on the aglycone.[1,2] However, these assays usually require the combined use of auxiliary enzymes, such as α-glucosidase or glucoamylase, in order to generate the chromophore or fluorophore from the glycosides. Furthermore, there are very few assays that directly and simply measure the hydrolysis of maltooligosaccharide derivatives spectroscopically.[3,4]

We have developed a simple assay system for α-amylase using a new approach based on supramolecular chemistry employing modified cyclodextrins. Cyclodextrins (CDs) are cyclic oligosaccharides, consisting of six, seven, and eight D-glucopyranose units for α-CD, β-CD, and γ-CD, respectively. CD can accommodate various hydrophobic organic compounds in its central cavity in aqueous solution.[5,6] Since the CD provides a hydrophobic environment within the central cavity, the spectroscopic properties of a chromophore molecule usually change upon uptake by the CD. On this basis, during the previous two decades, many chromophore-modified CDs have been synthesized as chemosensors for detecting a variety of organic compounds.[7–12] These types of CD-based chemosensors transduce the binding of guest molecules into spectroscopic signals through the guest-induced positional change of the chromophores, mostly from inside to outside the cavity. If the hydrophobic CD cavity that accommodates the fluorophore is disrupted by α-amylase, the fluorophore is then exposed to the hydrophilic aqueous solution resulting in a decrease in fluorescence intensity. In this paper, we report a direct and simple assay system for α-amylase using the change of fluorescence intensity of a modified CD by hydrolysis of the CD ring. The assay method can be used to analyze clinical samples since native β-CD and γ-CD are hydrolyzed by human salivary and pancreatic α-amylase at appreciable rates.[13]

2. Results and discussion

2.1. Designs of NBD-amine-modified CDs

We prepared CD derivatives bearing a 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD-amine) moiety as artificial

Keywords: Cyclodextrin; α-Amylase; Fluorescence; Enzyme assay.
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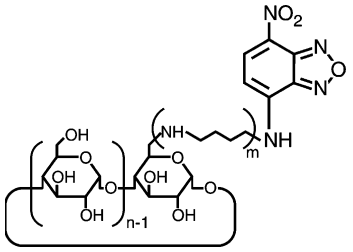

	m = 0	m = 1
n = 7	**NC0βCD**	**NC4βCD**
n = 8	**NC0γCD**	**NC4γCD**

Chart 1. Structures of NBD-amine-modified cyclodextrins.

substrates for the development of an α-amylase assay. NBD-amine displays the interesting property of fluorescing weakly in water and strongly in organic solvents, membranes or hydrophobic environments. Moreover, it has a long excitation wavelength (~480 nm) and long emission wavelength (~560 nm) in water, which avoids the interference from the bio-matrixes.[14,15] We selected β-CD and γ-CD as substrate skeletons, since the respective cavity size is compatible with the NBD-amine moiety. To investigate the effect of self-inclusion depth on the variation of the fluorescence intensity by the hydrolysis reaction, we prepared two types of CD derivatives using different spacers between the NBD-amine moiety and the CD. This allowed us to vary hydrophobic environment around the fluorophore. In one derivative, the C6 hydroxy group of the glucose unit was substituted with the amino group of the NBD-amine moiety without an alkyl spacer. The second derivative contained an amino-tetramethylene spacer, which sufficiently allowed the NBD-amine moiety in a self-inclusion state. The CD derivatives based on β-CD were named **NC0βCD** and **NC4βCD**, and contained, respectively, no spacer and the amino-tetramethylene spacer. Likewise, the CD derivatives based on γ-CD were named **NC0γCD** and **NC4γCD**, and contained, respectively, no spacer and the amino-tetramethylene spacer (Chart 1).

2.2. Absorption spectra of NBD-amine-modified CDs

Aspergillus oryzae α-amylase was chosen as the model enzyme in this study because it was known to hydrolyze CDs and modified CDs.[16–18] Absorption spectra of all the NBD-amine-modified CD derivatives (10 μM) in the presence of the α-amylase were monitored at regular intervals over a period of 30 min in 200 mM acetic acid/sodium acetate buffer (pH 5.5) containing $CaCl_2$ (1×10^{-2} M) at 25 °C. The absorption spectra of **NC4γCD** are shown in Figure 1. The absorption intensities of all the NBD-amine-modified CDs increased upon addition of the α-amylase (0.3 mg/ml for **NC0βCD**, 3 mg/ml for **NC4βCD**, and 0.05 mg/ml for **NC0γCD** and **NC4γCD**). Since the molar absorption coefficients (ε) of the NBD-amine derivatives increase with increasing solvent polarity, [15] this result indicates that the hydrolysis of the CD ring liberates the NBD-amine moiety from the hydrophobic CD cavity to the bulk water solution. The amount of the α-amylase required to cause spectral changes within 30 min was much greater for the β-CD derivatives than for the γ-CD derivatives. It corresponds with results of a previous report that the rate of hydrolysis by *A. oryzae* α-amylase was of the order of γ-CD > β-CD.[13] Isosbestic points were observed for **NC0βCD**, **NC0γCD**, and **NC4γCD** at 505, 508, and 506 nm, respectively. The presence of the isosbestic point indicates that there are two conditions of the NBD-amine derivatives, that is, covered with and uncovered with CD. The wavelength at the isosbestic point was chosen as the excitation wavelength for fluorescence measurements. Although an isosbestic point for **NC4βCD** was not observed, 505 nm was chosen as the excitation wavelength, since the variation in peak intensity was comparatively small.

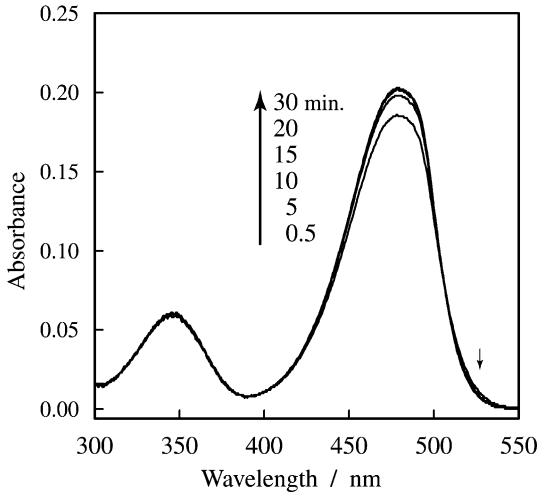

Figure 1. Changes of UV–vis spectra of **NC4γCD** in the presence of *Aspergillus oryzae* α-amylase over a period of 30 min in 200 mM acetic acid/sodium acetate buffer (pH 5.5, $[CaCl_2] = 1 \times 10^{-2}$ M) at 25 °C. [**NC4γCD**] = 10 μM. [α-amylase] = 0.05 mg/ml.

2.3. Fluorescence spectra of NBD-amine-modified CDs

Fluorescence spectra of all the NBD-amine-modified CDs (5 μM) show the emission bands with peaks around 565, 556, 566, and 566 nm for **NC0βCD**, **NC4βCD**, **NC0γCD**, and **NC4γCD**, respectively. The fluorescence intensity of the four NBD-amine-modified CD derivatives diminished upon addition of the α-amylase, indicating a decrease in hydrophobicity around the NBD-amine moiety induced by the hydrolysis of the CD ring. Change in the fluorescence spectrum of **NC4γCD** is shown in Figure 2. We also investigated the time course of the fluorescence intensity of each NBD-amine-modified CD in the presence of the same concentration of the α-amylase (0.02 mg/ml). $\Delta I/I_0$ for the four NBD-amine-modified CD derivatives ($\Delta I = I - I_0$, where I_0 denotes the initial fluorescence intensity and I denotes the fluorescence intensity after addition of the α-amylase) against time are shown in Figure 3. The variation of $\Delta I/I_0$ was greatest for **NC4γCD** and smallest for **NC0βCD**. This result is presumably due to difference in the rate of hydrolysis of CD by *A. oryzae* α-amylase (γ-CD > β-CD).

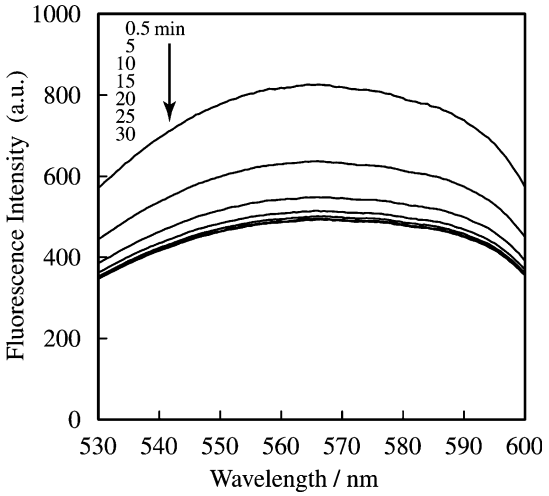

Figure 2. Changes in fluorescence spectra of **NC4γCD** in the presence of *Aspergillus oryzae* α-amylase over a period of 30 min in 200 mM acetic acid/sodium acetate buffer (pH 5.5, $[CaCl_2] = 1 \times 10^{-2}$ M) at 25 °C. [**NC4γCD**] = 5 μM. [α-amylase] = 0.03 mg/ml. The excitation wavelength was 506 nm.

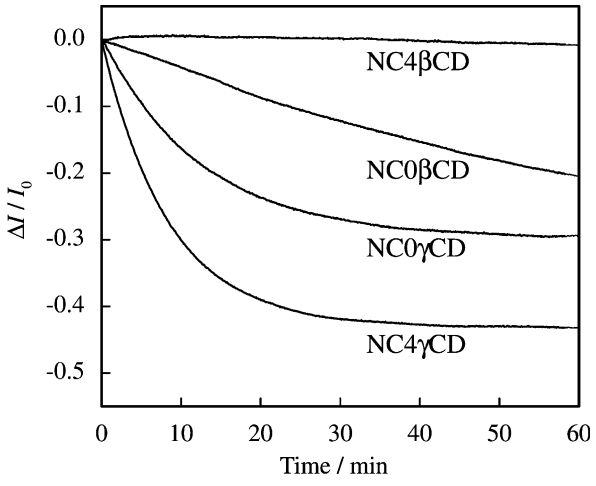

Figure 3. Time courses of fluorescence intensity of NBD-amine-modified CDs in the presence of *Aspergillus oryzae* α-amylase. [NBD-amine-modified CD] = 5 μM. [α-amylase] = 0.02 mg/ml. The excitation wavelength was 505 nm for **NC0βCD**, 505 nm for **NC4βCD**, 508 nm for **NC0γCD**, and 506 nm for **NC4γCD**. The emission wavelength was 565 nm for **NC0βCD**, 556 nm for **NC4βCD**, 566 nm for **NC0γCD**, and 566 nm for **NC4γCD**.

Furthermore, difference in fluorescence intensity of each derivative in the absence of the α-amylase (i.e., I_0) could have contributed to this result. The fluorescence intensities between same-sized CD derivatives are dependent on the inclusion depth of the NBD-amine moiety into the hydrophobic CD cavity. The NBD-amine moiety of **NC4γCD**, having an amino-tetramethylene spacer, might be more deeply buried within the hydrophobic CD cavity than that of **NC0γCD** that has no spacer. To investigate the effect of the depth of the NBD-amine moiety in the self-inclusion state on the fluorescence intensity, the saturated $\Delta I/I_0$ values were estimated by following the hydrolysis reaction of NBD-amine-modified CD (5 μM) for 1 h upon addition of sufficient α-amylase to completely degrade the CD derivatives (0.1 mg/ml for **NC0βCD**, 3 mg/ml for **NC4βCD**, and 0.02 mg/ml for **NC0γCD** and **NC4γCD**). The saturated $\Delta I/I_0$ value was −0.372, −0.382, −0.294, and −0.432 for **NC0βCD**, **NC4βCD**, **NC0γCD**, and **NC4γCD**, respectively. Each saturated $\Delta I/I_0$ correlated with the effect of hydrophobic CD cavity. The change in fluorescence intensity was greatest for **NC4γCD**, indicating that the NBD-amine moiety of this CD derivative is located in the most hydrophobic environment in the self-inclusion state. Thus, we selected **NC4γCD** as the most sensitive substrate for the α-amylase assay.

2.4. Analyses of the reaction product of NBD-amine-modified CDs hydrolyzed by *A. oryzae* α-amylase

Aliquots of the **NC4γCD** reaction mixture were taken at regular intervals. The reaction was quenched by addition of 1 M HCl aq and the samples were then assayed by HPLC monitoring at 450 nm (Fig. 4). Each HPLC peak was assigned by MALDI-TOF MS. The intensity of the peak corresponding to **NC4γCD** diminished as the reaction proceeded, with the concomitant appearance of new peaks. The intensity of the new peaks increased with the passage of the reaction. The peaks with a retention time of 14.4 and 15.2 min were assigned by MALDI-TOF MS as NBD-amine-appended maltohexaose and NBD-amine-appended maltopentaose, respectively. Although the precise structures of these products have not been determined, the molecular weight data suggest that **NC4γCD** is hydrolyzed to oligosaccharide derivatives by the α-amylase. This result indicates that the decrease of the fluorescence intensity

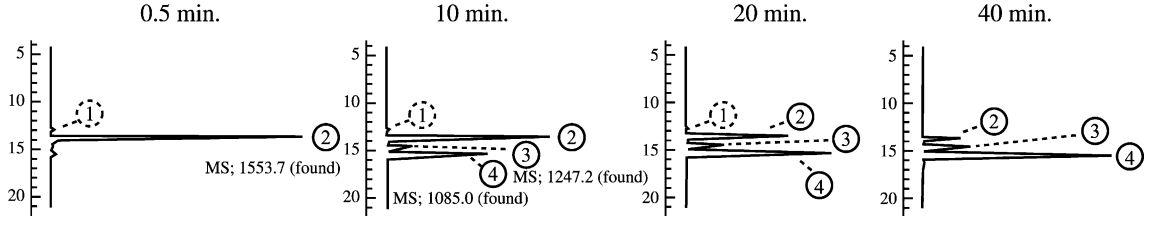

Figure 4. Analyses of the reaction products of **NC4γCD** hydrolyzed by *Aspergillus oryzae* α-amylase assayed by HPLC (ODS column, gradient elution from 5% to 30% acetonitrile for 30 min). The reaction was performed in 200 mM acetic acid/sodium acetate buffer (pH 5.5, $[CaCl_2] = 1 \times 10^{-2}$ M) at room temperature. [**NC4γCD**] = 100 μM. [α-amylase] = 0.01 mg/ml. The molecular weight of the peak **1** (12.8 min) could not be detected by MALDI-TOF MS. The molecular weight assigned by MALDI-TOF MS; the peak **2** (13.5 min): *m/z* found 1553.7 (calcd for [**NC4γCD** + Na]$^+$, 1553.4); the peak **3** (14.4 min): *m/z* found 1247.2 (calcd for [**NC4(Glu)$_6$** + Na]$^+$, 1247.2); the peak **4** (15.2 min): *m/z* found 1085.0 (calcd for [**NC4(Glu)$_5$** + Na]$^+$, 1084.9). **NC4(Glu)$_n$** represents 6-deoxy-6-(4-NBD-aminobutylamino)-oligosaccharides consisting of n glucose units.

of **NC4γCD** by the addition of the α-amylase is induced by hydrolysis of the CD ring. Similar results were also obtained in the case of other NBD-amine-modified CDs.

2.5. Dependence of the initial rate of the hydrolysis reaction of NC4γCD on the concentration of *A. oryzae* α-amylase

The dependence of the initial rate of hydrolysis of **NC4γCD** on the concentration of the α-amylase was studied. The initial rate of the reaction was estimated by the change of the fluorescence intensity of **NC4γCD**. The averages of five rate determinations were used to obtain a calibration curve and the standard deviation of each rate is shown as an error bar in Figure 5. An approximately linear relationship was obtained between the initial rate of the hydrolysis reaction, monitored by the fluorescence intensity, and the concentration of the α-amylase (0–7 μg/ml) (Fig. 5). Therefore, the concentration of the α-amylase can be easily monitored using this working curve.

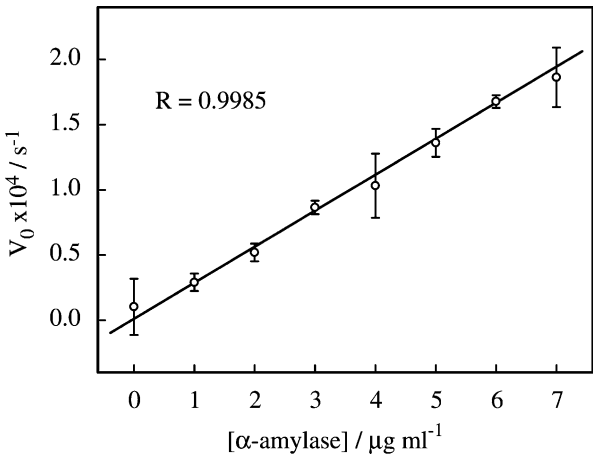

Figure 5. Calibration curve for *Aspergillus oryzae* α-amylase using the decrease of fluorescence intensity induced by hydrolysis reaction of **NC4γCD**. [**NC4γCD**] = 5 μM. The excitation wavelength was 506 nm; the emission wavelength was 566 nm. V_0 is the initial rate of variation of $\Delta I/I_0$ per second.

2.6. Influence of organic compounds on the fluorescence intensity of NC4γCD

Chromophore-modified CD may also act as a chemosensor for detecting a variety of organic compounds.[7–12] So, the presence of a specific organic compound in an assay solution might cause a change in the fluorescence intensity of NBD-amine-modified CDs. To explore this further, the sensitivities of NBD-amine-modified CDs (5 μM) for various organic compounds were evaluated using the variation in fluorescence intensity ($\Delta I/I_0$; $\Delta I = I - I_0$, where I_0 and I denote the fluorescence intensities in the absence and presence of a guest, respectively). The concentration of organic compound was fixed at 10 μM, which was sufficient to change the fluorescence intensity of a conventional CD-based chemosensor (Fig. 6). We tested 15 different organic compounds, which are typical guests for β-CD and γ-CD in the sensitivity study.[7–12] **NC0βCD**, **NC4βCD**, and **NC0γCD** were sensitive to all these organic compounds, whereas **NC4γCD** was not sensitive to every guest. This result indicates that, unlike the other NBD-amine-modified CDs, the fluorescence intensity of **NC4γCD** is unaffected by organic compounds in the assay mixture. Therefore, we conclude that **NC4γCD** is the most appropriate substrate for the measurement of *A. oryzae* α-amylase activity, because it is sensitive to the enzyme but not to the guest compounds.

3. Conclusion

A novel assay for α-amylase was developed using supramolecular chemistry. NBD-amine-modified CDs were tested as potential substrates for the assay of α-amylase. **NC4γCD** was the most sensitive substrate for *A. oryzae* α-amylase and not sensitive to organic compounds. A calibration curve of variation of fluorescence intensity versus the α-amylase concentration was obtained. The sensitivity of our system (> 1 μg/ml) is enough for clinical samples, because concentration of α-amylase in a human serum is 0.5–1.6 mg/ml and it is known that γ-CD can be hydrolyzed by human salivary and pancreatic

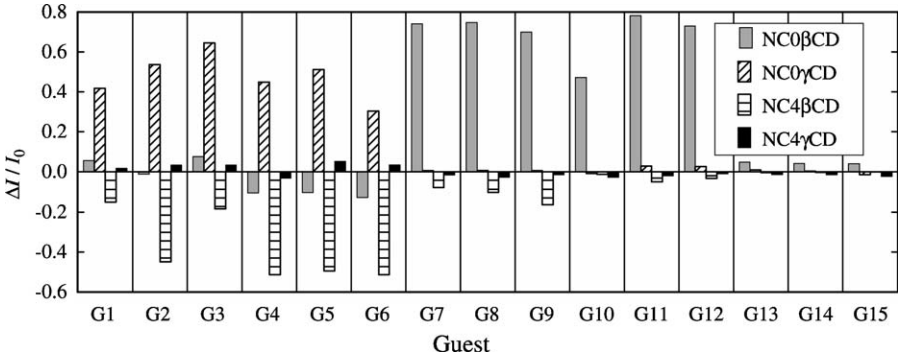

Figure 6. Sensitivity parameters ($\Delta I/I_0$) of NBD-amine-modified CDs (5 μM) for various guests. λ_{ex} was 510 nm for **NC0βCD**, 500 nm for **NC0γCD**, 434 nm for **NC4βCD** and 431 nm for **NC4γCD**. λ_{em} was 569.5 nm for **NC0βCD**, 566 nm for **NC0γCD**, 539.5 nm for **NC4βCD** and 556 nm for **NC4γCD**. [Guest] = 10 μM. G1, cholic acid; G2, chenodeoxycholic acid; G3, deoxycholic acid; G4, hyodeoxycholic acid; G5, lithocholic acid; G6, ursodeoxycholic acid; G7, 1-adamantanol; G8, 2-adamantanol; G9, 1-adamantane carboxylic acid; G10, 1-adamantanamine; G11, (+)-borneol; G12, (−)-borneol; G13, nerol; G14, geraniol; G15, cyclohexanol.

α-amylases at appreciable rate. This robust assay system could be useful in a variety of different practical applications including the analysis of clinical samples. The investigation for application of our assay system to practical settings is now under way.

4. Experimental

4.1. Material

CDs were kindly donated by Nihon Shokuhin Kako Co., Ltd, and were used without further purification. *A. oryzae* α-amylase and 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) and other reagents were purchased from Sigma–Aldrich Co., Tokyo Kasei Kogyo Co., Ltd, and Wako Pure Chemical Industries, Ltd, respectively, and were used without further purification. Deuterium oxide for NMR measurements was obtained from Merck Co.

4.2. Measurement

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 concd NH_3 aq:ethyl acetate:2-propanol:water, 1:3:5:4) was carried out with silica gel F_{254} (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.

4.3. Synthesis of mono-6-(4-aminobutylamino)-6-deoxy-γ-CD

A solution of mono-6-*O*-(2-naphthalenesulfonyl)-γ-CD[19] (729 mg, 0.5 mmol) in DMF (5.3 ml) was added dropwise to a solution of 1,4-diaminobutane (4.41 g, 50 mmol) in DMF (1 ml) and the resulting mixture was stirred at 70 °C for 1 h. The solution was then poured into acetone (450 ml), and the precipitate was collected and dried in vacuo overnight, to give 619 mg of crude product. The crude product was dissolved in water and purified by column chromatography on CM Sephadex C-25. After removal of impurities by elution with water, the fractions containing the desired product were obtained with a gradient elution of ammonia solution from 1 to 2 M. The fractions were concentrated under reduced pressure. Lyophilization of the product gave a white powder (567 mg, yield 83.0%).

The product was characterized by TLC (*n*-butanol:ethanol:water, 5:4:3, $R_f = 0.00$; concd NH_3 aq:ethyl acetate:2-propanol:water, 1:3:5:4, $R_f = 0.03$) and MALDI-TOF MS (*m*/*z* 1406.6; calcd for $[M+K]^+$, 1406.4).

4.4. Syntheses of NC0βCD, NC4βCD, NC0γCD, and NC4γCD

NBD-Cl (28.2 mg, 141 μmol) was added to a solution of mono-6-amino-6-deoxy-*β*-cyclodextrin (NH_2–β-CD)[20] (20.0 mg, 17.6 μmol) in DMF (2.0 ml)/methanol (2.5 ml) containing triethylamine (6.1 μl, 44 μmol). The reaction mixture was stirred at room temperature for 4.5 h. The solution was then poured into acetone (140 ml), and the precipitate was collected and dried in vacuo overnight, to give 18.6 mg of the crude product of **NC0βCD**. **NC4βCD**, **NC0γCD**, and **NC4γCD** were synthesized by the reaction of NBD-Cl with mono-6-(4-aminobutylamino)-6-deoxy-β-CD,[21] mono-6-amino-6-deoxy-γ-CD,[20] and mono-6-(4-aminobutylamino)-6-deoxy-γ-CD, respectively, in a DMF/methanol solution containing triethylamine in the same manner as **NC0βCD**. These crude products were purified by reverse-phase HPLC. The final products were obtained as a yellow powder (**NC0βCD**: 12.6 mg, yield 55.2%; **NC4βCD**: 18.5 mg, yield 40.8%; **NC0γCD**: 8.0 mg, yield 9.6%; **NC4γCD**: 6.1 mg, yield 13.6%).

The products were characterized by TLC (*n*-butanol:ethanol:water, 5:4:3, $R_f = 0.51$ for **NC0βCD**, 0.00 for **NC4βCD**, 0.45 for **NC0γCD**, 0.00 for **NC4γCD**; concd NH_3 aq:ethyl acetate:2-propanol:water, 1:3:5:4, $R_f = 0.54$ for **NC0βCD**, 0.55 for **NC4βCD**, 0.35 for **NC0γCD**, 0.33 for **NC4γCD**) and ^{1}H NMR (D_2O, 500 MHz) : **NC0βCD**: δ 4.89–5.13 (m, 7H, H-1), 6.43 (br s, 1H, aromatic), 8.45 (d, 1H, aromatic); **NC4βCD** : 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); **NC0γCD**: δ 4.93–5.22 (m, 8H, H-1), 6.36 (d, 1H, aromatic), 8.42 (d, 1H, aromatic); **NC4γCD**: 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 **NC0βCD**: *m*/*z* 1320.9 (calcd for $[M+Na]^+$, 1320.1), **NC4βCD**: *m*/*z* 1390.9 (calcd for $[M+Na]^+$, 1391.2), **NC0γCD**: *m*/*z* 1482.1 (calcd for $[M+Na]^+$, 1482.2), **NC4γCD**: *m*/*z* 1552.7 (calcd for $[M+Na]^+$, 1553.4).

Acknowledgments

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