Effect of Spacer Length in Pyrene-Modified-Phenylboronic Acid Probe/CyD Complexes on Fluorescence-based Recognition of Monosaccharides in Aqueous Solution

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Abstract

Chemical sensing of saccharides is of importance in the diagnosis of diabetes. Various enzymatic sensors have been developed, but their heat or pH instability issue needs be resolved. In this regard, the development of artificial saccharide sensors with high stability is attracting attention. Herein, we designed a heat- and pH-stable supramolecular inclusion complex system composed of cyclodextrin (CyD) as host and phenylboronic acid (PB) probe possessing pyrene as fluorescent guest. Several probes possessing alkyl spacers having various lengths between the PB and the pyrene moiety, Cn-APB (n = 1–4), were newly synthesized and evaluated with respect to their monosaccharide recognition ability on the basis of the fluorescence response through the cyclic esterification of monosaccharide and PB. These Cn-APB/CyD supramolecular inclusion complexes exhibited selective fluorescence response towards fructose in aqueous solution based on the photo-induced electron transfer mechanism. The spacer length of the alkyl group in Cn-APB significantly affected the affinity for saccharides. With respect to the complex between C4-APB and PB-modified CyD (3-PB-γ-CyD), it was found that the supramolecular inclusion complexes had high selectivity for glucose with significant fluorescence enhancement. These results indicate that the lengths of the alkyl spacers in the probe molecules are important to control the recognition of saccharides in aqueous solution.
Introduction

Saccharides are one of the essential biological molecules for the metabolism and maintenance of cell structure. Because of their important properties, much effort has been devoted to the development of simple methods that can selectively recognize saccharides in aqueous solution. Phenylboronic acids (PBs) form reversible covalent bonds with cis-1,2- and cis-1,3-diol-containing molecules, such as saccharides, generating five- and six-membered cyclic boronic esters in alkaline aqueous solution.\textsuperscript{1,2} Because of this unique property, PBs have been utilized in the development of artificial saccharide sensors.\textsuperscript{3-9} The binding constant of PBs for monosaccharides follows the order of fructose > galactose > mannose > glucose.\textsuperscript{2} In general, chemical probes that use PB have weak affinity and low selectivity for saccharides. Many researchers have examined molecular recognition in biological systems as it offers an important clue to solve these problems. To enhance binding strength, the synergistic effect of simultaneous multiple binding interactions has been utilized.\textsuperscript{10-13} In fact, enzymes or antibodies can strongly bind their target molecules because of the multiple binding interactions.

The versatile design of chemical sensors based on supramolecular chemistry is another approach to construct novel saccharide sensors.\textsuperscript{14,15} Previously, we designed a supramolecular inclusion complex system, namely, cyclodextrin (CyD) includes PB probe having pyrene as the fluorescent moiety.\textsuperscript{16} This system exhibited selective monosaccharide recognition ability in water with fluorescence response based on the photo-induced electron transfer (PET) from pyrene donor to PB acceptor.\textsuperscript{17} We also reported that the PB azoprobe/\textgamma-CyD complex showed high glucose selectivity in water by forming a 2:1 inclusion complex of PB azoprobe with \textgamma-CyD.\textsuperscript{18} Those results
indicated that the control of the molecular assembly of PB probe is a key factor to enhancing the selectivity for saccharides.

The interaction between PB probe and CyD is important to control the molecular assembly. We previously reported that differences in the fluorescent moiety of PB probes were responsible for the saccharide selectivity changes. In this study, we developed probes possessing alkyl spacers of various lengths between PB and pyrene, Cn-APB (Fig. 1, n = 1–4) and evaluated their saccharide recognition function on the basis of their fluorescence responses.

[Fig. 1]

**Experimental**

*Reagents and chemicals*

4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate (DMT-MM), pinacol, sodium hydroxide, hydrochloric acid, β-CyD, γ-CyD, tetrahydrofuran (THF), methanol, lithium aluminum hydride, diethyl ether, dichloromethane, sodium sulfate, magnesium sulfate, sodium azide, dimethylformamide (DMF), ammonia solution, sodium hydrogen carbonate, benzene, fructose, glucose, and galactose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4-Carboxyphenylboronic acid, triethylamine, methanesulfonyl chloride, triphenylphosphine, and oxalyl chloride were purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). 1,4-Dioxane, chloroform, acetonitrile, sulfuric acid, and acetone were purchased from Kanto Chemical, Co., Inc. (Tokyo, Japan). Methanol-\(d_4\), chloroform-\(d\), DMSO-\(d_6\), 1-pyreneacetec acid, and 1-pyrenebutyric acid were purchased from Sigma-Aldrich Japan, Co., LLC (Tokyo, Japan). 1-Pyrenebutylamine was
purchased from Toronto Chemical Research Inc. (Toronto, USA). All other organic solvents and reagents were commercially available with guaranteed grades and used without further purification. Water was doubly distilled and deionized by a Milli-Q water system (WG222, Yamato Scientific Co., Ltd., Tokyo, Japan and Autopure WR-600G, Merck Millipore, MA, USA) before use.

**Apparatus**

$^1$H NMR spectra were measured with a Lambda GX-500 (JEOL Ltd., Tokyo, Japan) at 300 K. Elemental analysis was performed with a PerkinElmer 2400 Series II CHNS/O Elemental Analyzer (PerkinElmer, Inc., MA, USA). Mass spectrometry was performed on a JMS-T100LC (JEOL, Ltd., Tokyo, Japan). All pH values were recorded with a Horiba F-52 pH meter (HORIBA, Ltd., Kyoto, Japan). UV-vis absorption spectra were measured with a Hitachi U-3900 UV-vis spectrophotometer (Hitachi High-Technologies, Co., Tokyo, Japan) equipped with a Peltier thermocontroller with a 10-mm quartz cell at 25°C. Fluorescence spectra were measured with a Hitachi F-7000 fluorescence spectrophotometer (Hitachi High-Technologies, Co., Tokyo, Japan) equipped with a Peltier thermocontroller with a 10-mm quartz cell at 25°C.

**Synthesis**

C1-APB and 3-PB-γ-CyD were synthesized as reported. Synthetic procedure of C2-APB, C3-APB, and C4-APB was shown in supporting information. Chemical structures of these compounds were shown in Fig. 2.

[Fig. 2]
**pH-fluorescence profile of Cn-APB/CyD complex**

To evaluate the saccharide recognition ability of Cn-APB/CyD complex, fluorescence spectral measurements were performed. Solutions containing Cn-APB (10 μM), CyD (0.5 mM), NaCl (100 mM), and H₃PO₄ (10 mM) were prepared and fluorescence spectra were measured at 25°C at various pH values in the absence or presence of saccharide (30 mM) with the addition of NaOHₐq.

**Fluorescence response of Cn-APB/CyD complex**

To evaluate the fluorescence response of Cn-APB/CyD complex toward saccharides, fluorescence spectral measurements were performed. Solutions containing Cn-APB (10 μM), CyD (0.5 mM), saccharides (30 mM), NaCl (100 mM), and H₃PO₄ (10 mM) were prepared and fluorescence spectra were measured at pH 10.5.

**pH-fluorescence profile of C4-APB/3-PB-γ-CyD complex**

To evaluate the saccharide recognition ability of C4-APB/3-PB-γ-CyD complex, fluorescence spectral measurements were performed. Solutions containing C4-APB (10 μM), 3-PB-γ-CyD (0.5 mM), NaCl (100 mM), and H₃PO₄ (10 mM) were prepared and fluorescence spectra were measured at 25°C at various pH values in the absence or presence of saccharide (30 mM) with the addition of NaOHₐq.

**Saccharide titration measurement of C4-APB/3-PB-γ-CyD complex**

To evaluate the saccharide response of C4-APB/3-PB-γ-CyD complex, fluorescence spectral measurements were performed. Solutions containing C4-APB (10 μM), CyD (0.5 mM), NaCl (100 mM), and H₃PO₄ (10 mM) were prepared and fluorescence spectra were measured at pH 10.5 with the addition of saccharides.
Results and Discussion

In order to evaluate the supramolecular inclusion complex formation and the fluorescence responses of the $Cn$-APB ($n = 1–4$)/β- or γ-CyD complexes, the pH-fluorescence profiles were measured in the presence of 30 mM fructose (Fig. 3). The addition of NaOH increased the fluorescence maximum at 375 nm for all combinations of probes and CyDs. The fluorescence emission at 375 nm is widely known as the monomer emission of pyrene. These results indicated that the $Cn$-APB/CyD complexes recognized fructose and showed monomer fluorescence emission under basic conditions. The fluorescence emission at 475 nm was increased only for the $C4$-APB/γ-CyD complex. The fluorescence emission at 475 nm is known as the dimer emission of pyrene. These results demonstrated that $C4$-APB formed 1:1 complex with β-CyD and 2:1 complex with γ-CyD.\textsuperscript{21} In addition, an appropriate length of the alkyl spacer ($n = 4$) was required for the dimer formation of the $Cn$-APB/γ-CyD complexes.

[Fig. 3]

In order to evaluate the saccharide recognition function of the $Cn$-APB/CyD complexes, the fluorescence intensity changes of the $Cn$-APB/β-CyD complexes ($n = 1$, 4) with pH in the presence or absence of saccharides were investigated (Fig. 4). Under acidic conditions, fluorescence emission of the $Cn$-APB/β-CyD complexes was not observed because PET from pyrene to boronic acid moiety was strongly promoted. Under basic conditions, boronic acid formed an anionic tetrahedral structure and reduced the electron acceptor function. Thus, PET from pyrene to boronic acid was inhibited and the fluorescence of $Cn$-APB was increased. The apparent pKa of $Cn$-APB shifted to a low pH region in the presence of saccharides compared with the case in the absence
of saccharides. When alkyl spacer of \textit{Cn-APB} was short, the effect of PET became much stronger, and thus the fluorescence intensity was reduced. On the other hand, another factor affecting the fluorescence intensity is the difference in binding ability of \textit{Cn-APB} with CyD based on the difference in spacer length; the fluorescence intensity decreases when the inclusion ability is low. For example, in the \(\gamma\)-CyD complex, the fluorescence intensity of dimer emission was reduced for \textit{Cn-APB} having short alkyl spacer. This is due to the steric hindrance of two probes inside \(\gamma\)-CyD by forming 1:1 complex with fructose in addition to the PET effect. From these reasons, the fluorescence intensities were strongly affected by the alkyl spacer length of \textit{Cn-APB} in CyD complexes.

[Fig. 4]

The apparent pK\textsubscript{a} values of the \textit{Cn-APB}/\(\beta\)-CyD complexes in the presence or absence of saccharides were obtained from the pH titration measurements (Table 1). The apparent pK\textsubscript{a} was decreased by the recognition of saccharides. The pK\textsubscript{a} values of the \textit{Cn-APB}/\(\beta\)-CyD complexes showed the largest decrease for fructose, indicating that the binding constant \((K_{LS}: \text{See Supporting information})\) for fructose is largest among those for the other saccharides. From the curve fitting analysis in Fig. 4, the apparent pK\textsubscript{a} was calculated and the binding constants for saccharides were determined from \(\Delta\text{pK}\textsubscript{a}\) (See Supporting information). The binding strength to saccharides determined by \(\Delta\text{pK}\textsubscript{a}\) was almost the same for those of our previous studies.\textsuperscript{21} The observed binding constants of the \textit{Cn-APB}/\(\beta\)-CyD complexes revealed that \textit{C4-APB} most strongly bound fructose, indicating that the hydrophobic interaction between the longest spacer of \textit{C4-APB} and CyD was important to induce strong saccharide binding.

[Table 1]

The fructose recognition function of the \textit{Cn-APB}/CyD complexes at pH 10.5 was
evaluated (Fig. 5). In the presence of β-CyD, monomer fluorescence intensity increased as the alkyl spacer length of Cₙ-APB increased. This result demonstrated that PET was suppressed as the alkyl spacer length was increased and C₄-APB/β-CyD formed a stable complex through the efficient hydrophobic interaction between Cₙ-APB and β-CyD with high monomer emission. On the other hand, in the presence of γ-CyD, Cₙ-APB showed the dimer emission in addition to the monomer emission. The fluorescence intensity of the dimer emission increased as the increase of alkyl spacer length, and it reached maximum for C₄-APB/γ-CyD. This result indicated that the large cavity of γ-CyD contributed to the stable dimer complexation between C₄-APB and γ-CyD. This result also revealed that the flexibility of spacer based on the alkyl chain length in the Cₙ-APB was important for the formation of the dimer complex within the γ-CyD cavity.

[Fig. 5]

To realize the multipoint interaction for saccharide recognition, PB-modified γ-CyD (3-PB-γ-CyD) was newly designed. Selective 2:1 interaction between PBs and glucose is expected for the C₄-APB/3-PB-γ-CyD complex.²⁰ The fluorescence intensity changes of the C₄-APB/3-PB-γ-CyD complex based on pH titration measurement are shown in Fig. 6. As expected, in the presence of glucose, the C₄-APB/3-PB-γ-CyD supramolecular inclusion complex exhibited the strongest fluorescence emission, indicating that the additional PB binding site of the C₄-APB/3-PB-γ-CyD complex was suitable for the stable 2:1 complex formation with glucose. In fact, no glucose selectivity was noted for unmodified CyD complexes with Cₙ-APB (Table 1). Among the Cₙ-APB/3-PB-γ-CyD complexes, the C₄-APB/3-PB-γ-CyD complex exhibited the highest fluorescence response for glucose (data not shown). The saccharide titration measurement for the C₄-APB/3-PB-γ-CyD complex was also examined (Fig. 7). I/I₀ is the ratio of the fluorescence intensities at 376 nm (pyrene monomer emission) before and
after the addition of saccharides. It was evident that $I/I_0$ was increased by glucose addition and decreased by fructose addition. These results demonstrated that C4-APB and 3-PB-γ-CyD formed a stable 2:1 complex with glucose as a result of the multipoint interaction in water. It was also found that the C4-APB/3-PB-γ-CyD complex was poorly recognized of fructose, which resulted in the decrease of the fluorescence intensity.

[Fig. 6]
[Fig. 7]

Conclusions

We have synthesized Cn-APB possessing different alkyl spacers ($n = 1–4$) and evaluated their saccharide recognition function on the basis of their fluorescence responses. The Cn-APB/CyD complexes exhibited fructose selectivity in water. The alkyl spacer length of Cn-APB was found to affect the saccharide binding function for the monomer emission, and strong dimer emission was observed for the C4-APB/γ-CyD complex on recognition of fructose. These results indicated that the hydrophobicity and the rigid structure are important factors for the design of the fluorescent supramolecular sensor. We also evaluated the saccharide recognition function of the C4-APB/3-PB-γ-CyD complexes. The C4-APB/3-PB-γ-CyD complex was found to exhibit glucose selectivity in water. These results demonstrated that C4-APB and 3-PB-γ-CyD formed a stable and rigid 2:1 complex with glucose as a result of the multipoint interaction in water. It became evident that controlling the alkyl spacer length of the boronic acid probe is an important factor for the selective recognition of saccharides in water.

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Supporting Information

$^1$H NMR and MS spectra are available in Supporting Information. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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Table 1  Apparent pKₐ and binding constants of Cn-APB/β-CyD complexes.

|       | None         | Glucose  | Fructose | Galactose |
|-------|--------------|----------|----------|-----------|
| C1-APB/β-CyD | pKₐ     | 8.23±0.11 | 7.65±0.10 | 6.14±0.08 | 7.38±0.10 |
|       | ΔpKₐ        | —        | 0.58±0.21 | 2.09±0.19 | 0.85±0.21 |
|       | Kₐₐ / M⁻¹   | —        | 93±79     | 4070±2250 | 203±146   |
| C2-APB/β-CyD | pKₐ     | 8.20±0.02 | 7.60±0.02 | 6.15±0.03 | 7.51±0.05 |
|       | ΔpKₐ        | —        | 0.60±0.04 | 2.05±0.05 | 0.69±0.07 |
|       | Kₐₐ / M⁻¹   | —        | 99±13     | 3710±453  | 130±28    |
| C3-APB/β-CyD | pKₐ     | 8.63±0.02 | 8.06±0.02 | 6.51±0.06 | 7.57±0.03 |
|       | ΔpKₐ        | —        | 0.57±0.04 | 2.12±0.08 | 1.06±0.05 |
|       | Kₐₐ / M⁻¹   | —        | —         | —         | —         |
| C4-APB/β-CyD | pKₐ     | 9.03±0.03 | 8.47±0.02 | 6.76±0.04 | 7.89±0.02 |
|       | ΔpKₐ        | —        | 0.56±0.05 | 2.27±0.07 | 1.14±0.05 |
|       | Kₐₐ / M⁻¹   | —        | 88±14     | 6170±1090 | 427±56    |
Figure Captions

Fig. 1  Chemical structure of Cn-APB/CyD complex.

Fig. 2  Chemical structures of Cn-APB (n = 1-4) and 3-PB-γ-CyD. (a) C1-APB, (b) C2-APB, (c) C3-APB, (d) C4-APB, (e) 3-PB-γ-CyD.

Fig. 3  Fluorescence spectral changes of Cn-APB/CyD complexes in 2% DMSO-98% water (v/v) by changing pH.  [Cn-APB] = 10 μM, [CyD] = 0.5 mM, [fructose] = 30 mM, [phosphate buffer] = 10 mM, [NaCl] = 100 mM, λ_ex = 352 (C1, 3, 4-APB), 350 nm (C2-APB).

Fig. 4  Fluorescence intensity changes of Cn-APB/β-CyD complexes in 2% DMSO-98% water (v/v) by changing pH.  [Cn-APB] = 10 μM, [β-CyD] = 0.5 mM, [saccharide] = 0 or 30 mM, [phosphate buffer] = 10 mM, [NaCl] = 100 mM, λ_ex = 352 nm.  The solid lines were drawn by curve fitting method based on the apparent pK_a analysis (S.I.).  Cn-APB/β-CyD were assumed to be a 1:1 inclusion complex and dissociated hydroxy moiety to bind with saccharides.

Table 1  Apparent pK_a and binding constants of Cn-APB/β-CyD complexes.

Fig. 5  (a) Monomer emission and (b) dimer emission of Cn-APB/CyD complexes in 2% DMSO-98% water (v/v).  [Cn-APB] = 10 mM, [CyD] = 0.5 mM, [fructose] = 30 mM, [NaCl] = 100 mM, [phosphate buffer] = 10 mM, adjusted to pH 10.5, λ_ex = 352 (C1, 3, 4-APB), 350 nm (C2-APB).
Fig. 6  Fluorescence intensity changes of **C4-APB/3-PB-γ-CyD** complexes in 2% DMSO-98% water (v/v) by changing pH.  
[C4-APB] = 10 μM, [3-PB-γ-CyD] = 0.5 mM, [saccharide] = 30 mM, [phosphate buffer] = 10 mM, [NaCl] = 100 mM, λ_ex = 350 nm.  The solid lines were drawn by curve fitting method based on the apparent pK_a analysis (S.I.).  **Cn-APB/3PB-γ-CyD** were assumed to be a 1:1 inclusion complex and dissociated hydroxy moiety to bind with saccharides.

Fig. 7  Changes of fluorescence intensity ratios (I/I_0) of **C4-APB/3-PB-γ-CyD** complexes in 2% DMSO-98% water (v/v) with the addition of saccharides.  
[C4-APB] = 10 μM, [3-PB-γ-CyD] = 0.5 mM, [saccharide] = 0–30 mM, [phosphate buffer] = 10 mM, adjusted to pH 10.5, [NaCl] = 100 mM, λ_ex = 350 nm.
Fig. 1 Chemical structure of $C_n$-APB/CyD complex.

Fig. 2 Chemical structures of $C_n$-APB ($n=1-4$) and 3-PB-$\gamma$-CyD. (a) C1-APB, (b) C2-APB, (c) C3-APB, (d) C4-APB, (e) 3-PB-$\gamma$-CyD.
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Graphical Index

Fructose Selectivity ↔ Glucose Selectivity