Fluorescence-based Indicator Displacement Assay for Phosphosugar Detection Using Zinc(II) Dipicolylamine-appended Phenylboronic Acid

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We have found that 6,7-dihydroxy-4-sulfonatemethylcoumarin more favorably binds to boron than to coordinated zinc(II) in Zn^{II}–dipicolylamine (DPA)-appended phenylboronic acid in water at neutral pH. By studying this novel property we have developed a fluorescence-based indicator displacement assay for the detection of phosphosugars in water.

The development of self-organized molecular sensors, achieved by linking molecular units through reversible interactions, is an intriguing interdisciplinary areas related to analytical chemistry and supramolecular chemistry.[1] An indicator displacement assay (IDA) is a valuable tool for analyte detection.[2] Zn^{II}–dipicolylamine (DPA)-appended receptors are often employed as a component with a metal centre in systems because the direct coordination of such indicators can induce a significant change in the photophysical properties of the systems.[3] Also, boronic acids in combination with catechol-fused dyes have been used in competitive binding assays of saccharides.[4] The study of reversible bond formation with such binding sites will enable us to develop effective IDAs. We have synthesized Zn^{II}–DPA-appended phenylboronic acid **1**·Zn, which forms an assembly with alizarin dye in aqueous media.[5] In this assembly, alizarin dye binds favorably to the Zn^{II}–DPA segment under neutral conditions prior to the competitive binding of the analyte. Interestingly, when alizarin dye is replaced with sodium 6,7-dihydroxycoumarin-4-methanesulfonate (**2a**),[6] the indicator is found to more favorably bind to boron than to the Zn^{II}–DPA moiety under neutral conditions. Since Zn^{II}–DPA has a strong affinity for phosphates,[7] the **2a**–**1**·Zn assembly can serve as a new IDA for phosphosugar detection. This approach is significant because phosphosugar-detectable IDAs are rare,[8] and phosphosugars are biologically important as seen in the pathway of glycolysis.[9] Therefore, assembly systems that can be used to determine the analyte at low concentrations in 100% water are intriguing research targets.

Figure 1 shows the data of fluorescence titration of **2a** (10 μM) with **1**·Zn in 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer containing 10 mM NaCl (pH 7.4) at 25 °C. The stepwise addition of **1**·Zn into the aqueous solution resulted in a significant increase in the fluorescence intensity ($\lambda_{ex} = 340$ nm). When phenylboronic acid-free Zn^{II}–DPA derivative, **3**·Zn, was used instead of **1**·Zn, the fluorescence intensity decreased (Figure S1).[10] These results indicate that **2a** binds to **1**·Zn via boronate esterification. Further assessment was carried out by ^{1}H NMR (Figure 2); when **1**·Zn and **2a** were mixed in D_2O containing 10 mM HEPES (pD 7.8), resonances arising from phenylboronic acid moiety and **2a**-based catechol were upfield shifted significantly, fully supporting the formation of a boronate ester.[11] The broad signals in Figure 2c

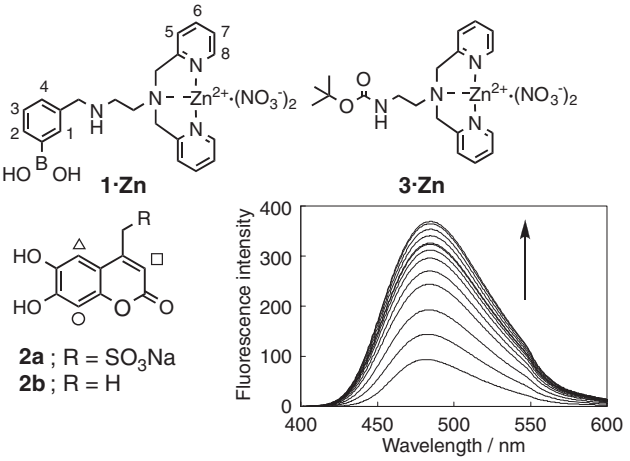

Figure 1. Change in fluorescence spectra of **2a** (10 μM) upon adding **1**·Zn in 10 mM HEPES buffer containing 10 mM of NaCl (pH 7.4) at 25 °C; [**1**·Zn] = 0, 10, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, and 400 μM. $\lambda_{ex} = 340$ nm.

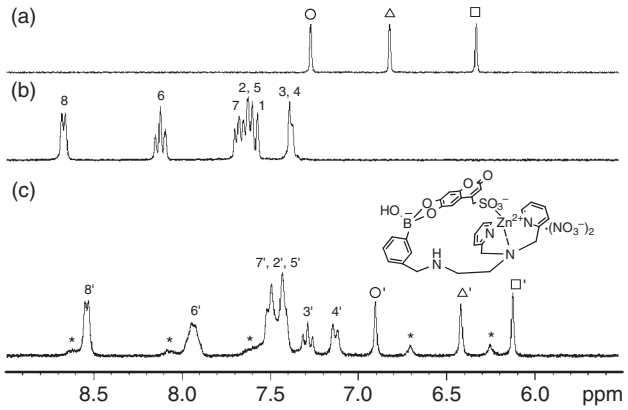

Figure 2. ^{1}H NMR spectra in D_2O with 10 mM HEPES at pD 7.8 (270 MHz, 24 °C). [**1**·Zn] = [**2**] = 5 mM, (a) **2**, (b) **1**·Zn, and (c) **2** plus **1**·Zn.

suggests that the aggregation equilibrates with **2a** and **1**·Zn. The relatively small peaks (*) in Figure 2c can be attributed to uncomplexed **1**·Zn and **2a**. When **1**·Zn was replaced with **3**·Zn under similar conditions, no perturbation of chemical shifts of **2a** and **3**·Zn was observed (Figure S2).[10] Thus, the upfield shifts of pyridyl protons (Figure 2c) can be ascribed to an intramolecular interaction, which may occur between the sulfonate site and Zn^{II}–DPA in **2a**–**1**·Zn under the NMR measurement conditions. The binding constant of **2a** with **1**·Zn has been estimated to be $(1.99 \pm 0.08) \times 10^4$ M^{-1} from the titration data shown in Figure S3;[10] from this binding constant, it can be com-

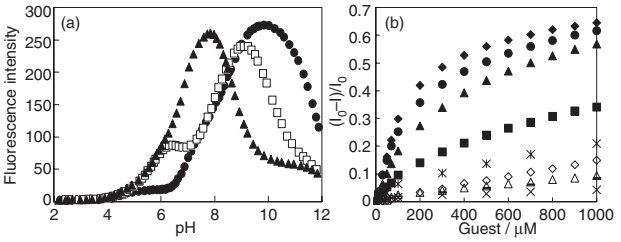

Figure 3. (a) Spectrofluorimetric pH-titrations of **2a** (●), **2a** plus **1**·Zn (▲), **2a** plus **1**·Zn with F6P (□) in H_2O containing 10 mM NaCl at 25 °C; [**2a**] = 10 μM; [**1**·Zn] = 100 μM, [F6P] = 500 μM, λ_{ex} = 340 nm, λ_{em} = 485 nm. (b) Plots of fluorescence intensity at 485 nm of **2** (10 μM) in the presence of **1**·Zn (100 μM) as a function of guest concentrations; F6P (◆), F1,6P (●), R5P (▲), G6P (■), D-fructose (*), G1P (◇), D-glucuronate (△), D-ribose (−), D-glucose (×). The measurements were carried out in 10 mM HEPES buffer containing 10 mM NaCl (pH 7.4) at 25 °C. I and I_0 denote the fluorescence intensity of **2a**–**1**·Zn in the presence and absence of guest, respectively. λ_{ex} = 340 nm.

puted that 74.3% of **2a** has been converted to **2a**–**1**·Zn assembly when 10 equiv of **1**·Zn (100 μM) is used.

The assembly **2a**–**1**·Zn has a great potential for application in the detection of phosphosugars in water. To estimate the function, the spectrofluorimetric pH titration of **2a** in water was carried out under several conditions, in which a solution containing excess acid was titrated with a standard base (Figure 3a). The fluorescence intensity of **2a** increased from pH 6.5 to 10 (●); this increase could be ascribed to the deprotonation of **2a**. However, in the presence of 10 equiv of **1**·Zn, the pH profile (▲) was different from that obtained in the presence of only **2a**; the fluorescence intensity at 485 nm increased up to pH 8, indicating the formation of the boronate ester of **2a** with **1**·Zn. Note that the presence of 50 equiv of D-fructose-6-phosphate (F6P) in the solution containing **2a** and **1**·Zn led to the formation of a somewhat complex pH profile (□); a plateau was observed from pH 6 to 7. The result can be interpreted by following equilibrium; **2a**–**1**·Zn + F6P ⇄ F6P–**1**·Zn + **2a**. The difference between the fluorescence intensity of **2a** and **1**·Zn in the presence and absence of F6P under neutral conditions is feasible to set up the conditions for applying **2a**–**1**·Zn assembly to phosphosugar sensing.

As a protocol for IDA, the fluorescence titration of **2a** in the presence of 10 equiv of **1**·Zn was conducted by increasing the concentration of guest species in the 10 mM HEPES buffer containing 10 mM NaCl at 25 °C. The change in the intensity at 485 nm was monitored upon the excitation at 340 nm. Several phosphosugars (F6P, D-ribose-5-phosphate (R5P), D-glucose-6-phosphate (G6P), D-fructose-1,6-diphosphate (F1,6P), α-D-glucose-1-phosphate (G1P)), D-glucuronate, and monosaccharides were employed as guests. As shown in Figure 3b, the addition of F6P into the solution of **2a** and **1**·Zn led to significant decrease in the fluorescence, indicating a dye-release process. During this process, F6P displaced indicator **2a** due to the synergistic effect of the competitive boronate esterification with the diol of the sugar moiety and the phosphate coordination in Zn^{II}–DPA. The estimation of the binding constant for **1**·Zn with F6P (K_a) is based on a competitive method.[12] The parameters Q and P are defined as follows: $P = [\mathbf{1}\cdot\mathrm{Zn}]_t - 1/(QK_I) - [\mathbf{2a}]_t/(Q+1)$ and $Q = [\mathbf{2a}]/[\mathbf{2a}\text{–}\mathbf{1}\cdot\mathrm{Zn}]$, where K_I is the binding constant for **2a** with **1**·Zn (vide supra). Figure S4[10] shows the relationship between Gt/P and Q (Gt denotes the total concentration of the guest), where the deviation was found to be large at $Q < 1.43$ ([F6P] = 200 μM). This deviation can be explained on the basis of the presence of an excess amount of **1**·Zn (10 equiv) as compared to **2a** in the solution. The desired K_a value has been calculated to be $(2.00 \pm 0.13) \times 10^4\ M^{-1}$, from linearity obtained ranging from 200 to 1000 μM (Figure S4[10]). Although R5P and F1,6P also exhibited similar quenching behavior, the use of G6P induced a somewhat low response in the fluorescence spectra. The K_a values of F1,6P and R5P estimated by a similar method are $(1.54 \pm 0.09) \times 10^4$ and $(1.41 \pm 0.10) \times 10^4\ M^{-1}$, respectively (Figure S4[10]). However, the K_a values of other guest species tested in this study could not be obtained because of the nonlinearity between Gt/P vs. Q; this nonlinearity indicates low reactivity of guest species at the boron site. Consequently, it appeared that the system showed a somewhat selective response toward phosphosugars in the following order: F6P, F1,6P, R5P > G6P > G1P. The higher affinity for F6P than G6P agrees with the tendency of boronic acid to form more stable complex with fructose than with glucose.[13] On the other hand, the poor response toward G1P in the fluorescence spectra may indicate that boronic acid hardly forms a stable six-membered ring with a *trans*-CH(OH)–CH(CH_2OH)-diol group of G1P.[14] Thus, the high affinity of **1**·Zn for F6P may be caused by cooperative interactions involving phosphate–Zn^{II}–DPA coordination and boronate esterification with the diol of saccharides.[15] Indeed, the addition of monosaccharides and D-glucuronate induced a poor response.

In conclusion, the proposed system is the first example of IDA with Zn^{II}–DPA for the selective fluorescence detection of phosphosugars in water at neutral pH.

References and Notes

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15 The binding profile using **2b** in MeOH was monitored by ESI-MS spectroscopy (Figure S6).[10]

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