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A Highly Selective and Sensitive Fluorescence Sensing System for Distinction between Diphosphate and Nucleoside Triphosphates

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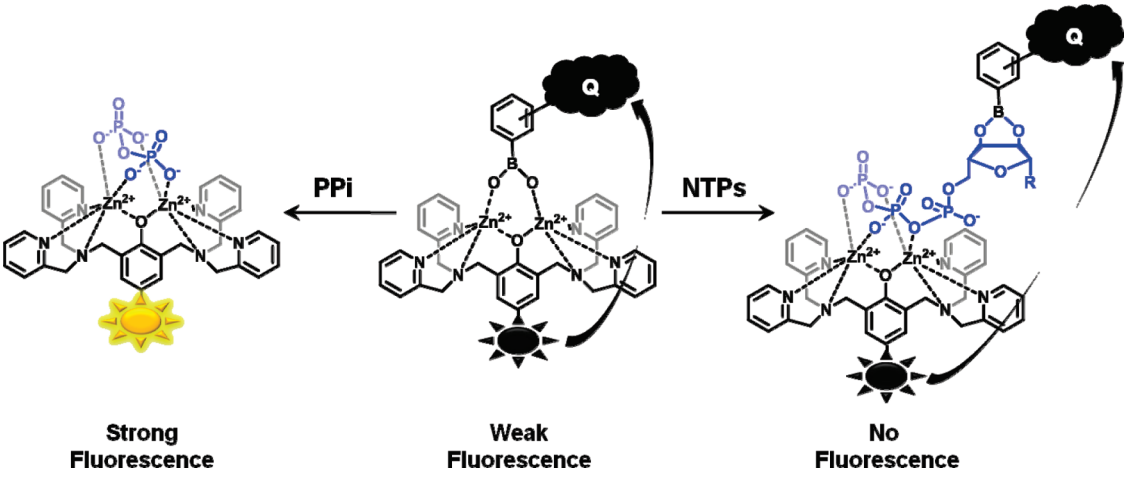

Among the numerous chemosensors available for diphosphate ($P_2O_7^{4-}$, PPi) and nucleoside triphosphates (NTPs), only a few can distinguish between PPi and NTPs. Hence, very few bioanalytical applications based on such selective chemosensors have been realized. We have developed a new fluorescence sensing system for distinction between PPi and NTPs based on the combination of two sensors, a binuclear Zn(II) complex (**1**·2**Zn**) and boronic acid (**BA**), in which one chemosensor (**1**·2**Zn**) shows signal changes depending on the PPi (or NTP) concentration, and the other (**BA**) blocks the signal change caused by NTPs; this system enables the distinction of PPi from NTPs and is sensitive to nanomolar concentrations of PPi. The new sensing system has been successfully used for the direct quantification of RNA polymerase activity.

Introduction

Recently, there has been a resurgence of interest in the design and development of diphosphate ($P_2O_7^{4-}$, PPi) sensors.[1−3] This is because PPi plays an important role in many biological processes by participating in enzymatic reactions.[4−6] In particular, PPi is released when nucleoside triphosphate (NTP) is incorporated into a growing DNA or RNA in a polymerase reaction.[5] PPi is also released when second messengers such

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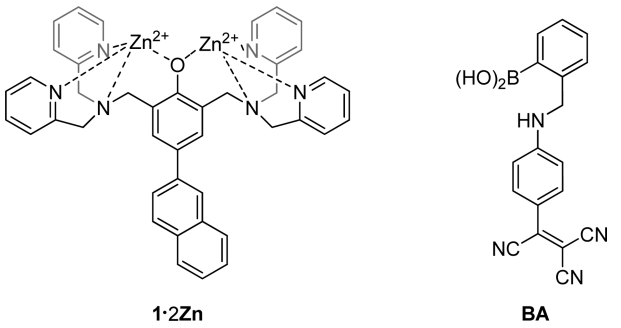
as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are synthesized from NTP.[6]

Till date, very few chemosensors that can distinguish PPi from NTPs have been reported, although numerous PPi sensors are available.[1g] Unfortunately, these chemosensors do not have sufficient affinity toward PPi, as is required for bioanalytical assays in which PPi usually is present in small amounts along with a large amount of NTPs.[4−6] On other hand, sensors that bind tightly to PPi suffer from poor selectivity for PPi (between PPi and NTPs).[3] Thus, for the successful real-time monitoring of enzymatic reactions that involve NTPs and the release of PPi, we must develop highly sensitive PPi sensors that can distinguish between PPi and NTPs.

A promising method for developing the aforementioned type of sensors involves the combination of two sensing systems; in this combination, one chemosensor shows signal changes depending on the PPi (or NTP) concentration, and the other blocks the signal change caused by NTPs. To the best of our knowledge, this is the first attempt toward the development of a chemosensing system that can distinguish between PPi and NTPs, by using two different chemosensors in a single solution.[7] On this basis, we designed a new PPi-sensing system that shows a drastic increase in the fluorescence emission intensity upon interacting with PPi and a decrease in the emission intensity upon interacting with NTPs. The advantage of this system is that it never misidentifies NTP as PPi, as fluorescence enhancement is observed only in the presence of PPi.

Further, our sensing system has been successfully used to monitor an RNA polymerase chain reaction. Accurate quantification of transcribed RNA is extremely important for a wide variety of molecular biology experiments such as those involving chemotherapy-response monitoring and measuring the transcription of gene-encoded therapeutic agents.[8] Currently, the amount of transcribed RNA is determined by using radioisotopes or fluorescent dyes.[9] However, the existing methods for RNA quantification, such as gel electrophoresis and treatment with RNase-free DNase, are laborious and time-consuming, whereas our sensing system enables the rapid and direct analysis of transcribed RNA.

SCHEME 1. Two Chemosensors Comprising the Sensing System (1·2Zn+BA)



1·2Zn **BA**

Results and Discussion

Design Concept. The sensing system comprises a dinuclear Zn(II) complex (**1·2Zn**) and boronic acid (**BA**), as shown in Scheme 1. **1·2Zn**, a fluorescent chemosensor for PPi, binds tightly with PPi in aqueous media; the corresponding binding constant in this case is 6.6 (±1.2) × 10^8 M^{-1}.[3b] **BA** is a chromogenic chemosensor for sugars whose absorption spectra overlap with the emission spectrum of **1·2Zn**.[10a]

The addition of NTPs to **1·2Zn** brings about a slight but measurable increase in the fluorescence intensity of the latter; in contrast, the addition of PPi leads to a significant increase in the fluorescence intensity.[3b] Thus, the increase in the fluorescence intensity of **1·2Zn** is not always due to the presence of PPi. However, in a mixture of **1·2Zn** and **BA**, fluorescence enhancement is expected to occur only when PPi is present. This is because the undesirable fluorescence increase caused by NTPs is probably prevented by the cooperative action of the two chemosensors in **1·2Zn**+**BA**. The **BA** molecule reacts with the ribose 2,3-*cis*-diol group in the NTPs to form a boronate ester, while the phenoxo-bridged dinuclear zinc complex in **1·2Zn** has strong affinity for phosphate derivatives.[3,11] Therefore, the NTPs are expected to undergo complexation with **1·2Zn** and **BA**, while PPi is expected to form a complex

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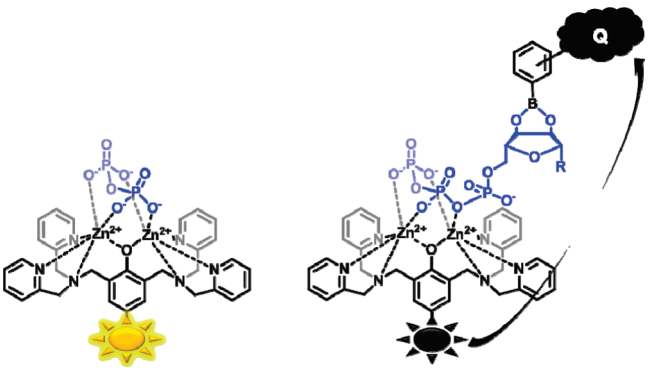


FIGURE 1. Design concept for discrimination between PPi and NTPs. Possible structures formed as a result of binding between **1**·2**Zn** and PPi (left) and between **1**·2**Zn**, **BA,** and NTPs (right).

only with **1**·2**Zn** (Figure 1). As a consequence, the NTP-induced fluorescence increase in **1**·2**Zn**+**BA** may be nullified by **BA** in the NTP complexes, as illustrated in Figure 1.

PPi Sensitivity. The effect of PPi on the emission spectra of **1**·2**Zn**+**BA** in aqueous CAPS buffer (CAPS = *N*-cyclohexyl-3-aminopropanesulfonic acid, 10 mM, pH 10.5) at 25 °C (Figure 2b) was examined. Job's plot for binding between **1**·2**Zn**+**BA** and PPi revealed a 1:1 binding stoichiometry (Supporting Information). The addition of PPi to **1**·2**Zn**+**BA** showed 8-fold increase in the fluorescence emission while saturation was reached at over 25 μM of PPi.

On the other hand, the addition of PPi to **1**·2**Zn** (10 μM) caused only a 6-fold increase in the emission intensity; in this case, fluorescence saturation was observed only after the addition of more than 15 μM of PPi (Figure 2a). The difference in Figure 2 panels a and b is due to the interaction between **1**·2**Zn** and **BA**. The association constant between **1**·2**Zn** and **BA** was estimated to be 2.3 ($\pm$0.5) $\times$ 10^5 M^{-1} by fluorescence titration. The Job plot for the binding between **1**·2**Zn** and **BA**, also showed a 1:1 binding stoichiometry (Supporting Information). MALDI-TOF mass data confirmed the 1:1 interaction between **1**·2**Zn** and **BA** (Supporting Information).

As illustrated in Scheme 2, the addition of **BA** to **1**·2**Zn** leads to a decrease in the fluorescence intensity because of the interaction between **BA** and **1**·2**Zn** (Figure 3). As a consequence, the background signal due to **1**·2**Zn**+**BA** becomes less intense than that due to **1**·2**Zn**, and this leads to an increase in the on/off ratio upon interaction of PPi (or NTPs) and **1**·2**Zn**+**BA**. On the other hand, **BA** moiety bound to **1**·2**Zn** in **1**·2**Zn**+**BA** can be displaced by PPi, which binds more strongly to **1**·2**Zn** than does BA.[12] Thus, since PPi competes with **BA** for binding with **1**·2**Zn**, a higher concentration of PPi is required for fluorescence saturation in the titration of **1**·2**Zn**+**BA**.

The disadvantage of the current sensing system is the unavoidable decrease in the intrinsic sensitivity of **1**·2**Zn**+**BA**

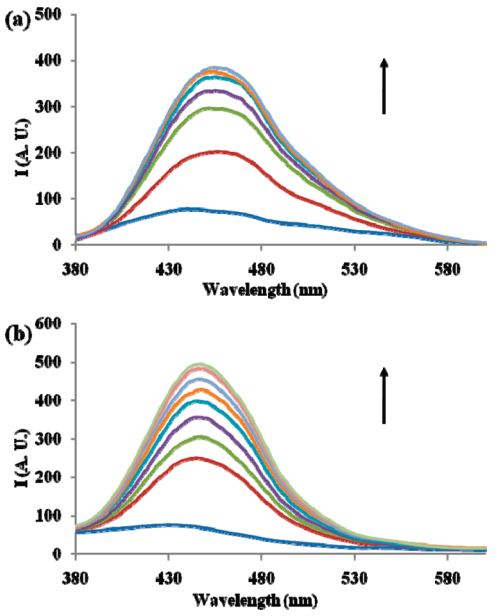
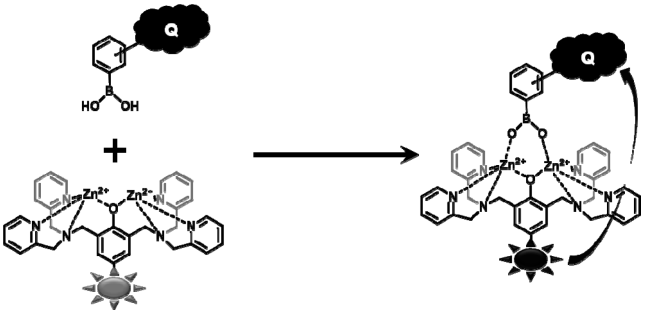


FIGURE 2. (a) Emission spectra (λ_{ex} = 317 nm) obtained after the addition of PPi solution (final concentrations: 0, 3, 5, 8, 10, 15, 20 μM) to an aqueous solution of 10 mM CAPS buffer (pH 10.5) containing **1**·2**Zn** (10 μM). (b) Emission spectra (λ_{ex} = 317 nm) obtained after the addition of PPi solution (final concentrations: 0, 3, 5, 8, 10, 15, 20, 25, 30 μM) to an aqueous solution of 10 mM CAPS buffer (pH 10.5) containing **1**·2**Zn**+**BA** (10 μM + 30 μM).

SCHEME 2. Interaction between 1·2Zn and BA



to PPi. This is because in our sensing system, **BA** is displaced from **1**·2**Zn** by PPi, and hence, the limit of detection (LOD) for PPi is increased.[13] However, the interaction between the two chemosensors (**1**·2**Zn** and **BA**) in this system does not have any significant effect on the LOD of **1**·2**Zn**+**BA** (10 μM + 30 μM). The LODs of **1**·2**Zn** (10 μM) and **1**·2**Zn**+**BA** (10 μM + 30 μM) for PPi are 32 and 50 nM, respectively. This result indicates that the disadvantage of the displacement approach for PPi sensing can be overcome or neglected in the case of **1**·2**Zn**+**BA**. This can be attributed to the reduced background signal intensity and the fact that **1**·2**Zn** has a higher binding affinity for PPi than for **BA**.

PPi Selectivity. The effect of various anions, including PPi and NTPs, on the emission intensity of **1**·2**Zn**+**BA** in aqueous CAPS buffer (10 mM, pH 10.5) at 25 °C was examined (Figure 4b). The addition of PPi and NTPs resulted in a notable change in the fluorescence intensity but addition of other anions did not (Supporting Information).

The addition of NTPs to **1**·2**Zn**+**BA** results in a decrease in the fluorescence intensity because the fluorescence emission

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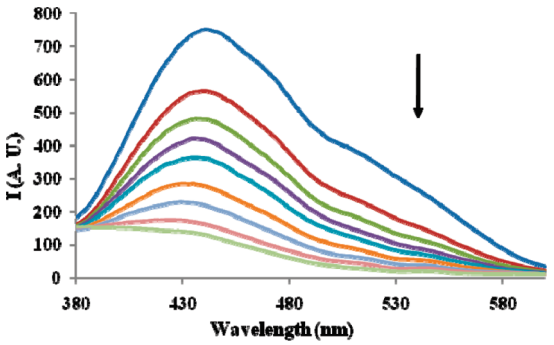


FIGURE 3. Emission spectra (λ_{ex} = 317 nm) obtained after the addition of **BA** solution (final concentrations: 0, 3, 5, 8, 10, 15, 20, 25, 30 μM) to an aqueous buffer solution (10 mM CAPS, pH 10.5) containing **1**·2**Zn** (10 μM).

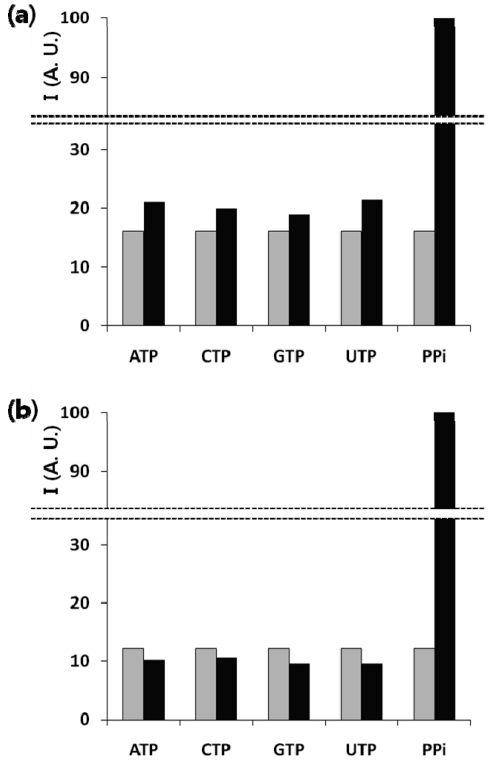


FIGURE 4. Relative fluorescence emission intensity (λ_{ex} = 317 nm) at λ_{max} in CAPS buffer solution (10 mM, pH 10.5) in the absence (gray bar) and presence (black bar) of various anions (10 μM, sodium salts): (a) **1**·2**Zn** (10 μM), (b) **1**·2**Zn**+**BA** (10 μM + 30 μM).

from the naphthyl group of **1**·2**Zn** is completely absorbed by **BA**; this absorption in turn is due to the binding of NTPs to **1**·2**Zn**+**BA**. However, the addition of NTPs to **1**·2**Zn** leads to a slight increase in the fluorescence intensity, as is already known. It is noteworthy that the **1**·2**Zn**+**BA** system never misidentifies NTPs as PPi because only the latter brings about an increase in the fluorescence intensity. As illustrated in Figure 1, the enhanced selectivity for PPi over NTPs is due to complex formation of NTPs with **BA** and **1**·2**Zn**. It is well-known that boronic acid has strong affinity for the cis diol groups in sugars such as glucose and fructose.[10] Similarly, boric acid has affinity for the cis diol group in the ribose moiety of

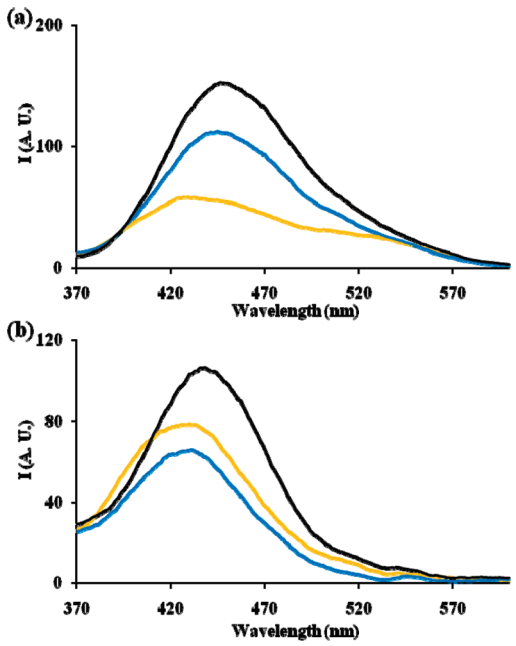
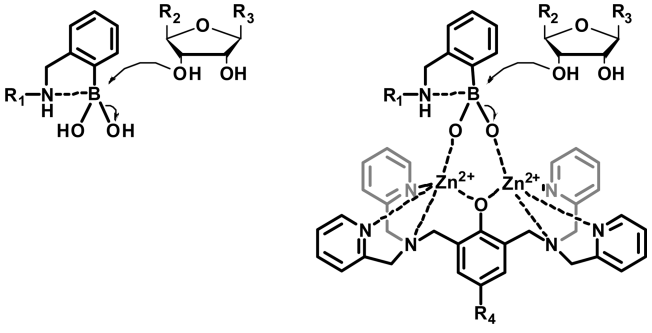


FIGURE 5. Fluorescence emission changes (λ_{ex} = 317 nm) observed upon the sequential addition of 40 μM NTPs (ATP + GTP + UTP + CTP, 10 μM each, blue line) and 1 μM PPi (black line) to an aqueous buffer solution (10 mM CAPS, pH 10.5) containing (a) **1**·2**Zn** (10 μM, yellow line) and (b) **1**·2**Zn**+**BA** (10 μM + 30 μM, yellow line).

SCHEME 3. Comparison of the Reactivities of BA Bound to 1·2Zn and Free BA



NTPs.[11] Additionally, the phenoxo-bridged dinuclear zinc complex in **1**·2**Zn** binds to the triphosphate group of NTPs.[3] Thus, NTPs form a complex with **BA** as well as with **1**·2**Zn**, as confirmed by MALDI−TOF mass analysis (Supporting Information). In the complex formed between NTPs and **1**·2**Zn**+**BA**, the fluorescence increase resulting from the binding of NTPs to **1**·2**Zn** is probably nullified by **BA**.

Sensing PPi in the Presence of NTPs. The detection of PPi in the presence of NTPs in an aqueous CAPS buffer solution (10 mM, pH 10.5) at 25 °C was examined (Figure 5). For bioanalytical applications, it is necessary to develop sensors that can selectively detect PPi in the presence of NTPs, as PPi usually coexists with excessive amounts of NTPs in a biological environment.[4−6]

As described above, the addition of NTPs to **1**·2**Zn**+**BA** caused a decrease in the fluorescence intensity, whereas the addition of NTPs to **1**·2**Zn** caused an increase in the fluorescence intensity (yellow line to blue line in Figure 5).

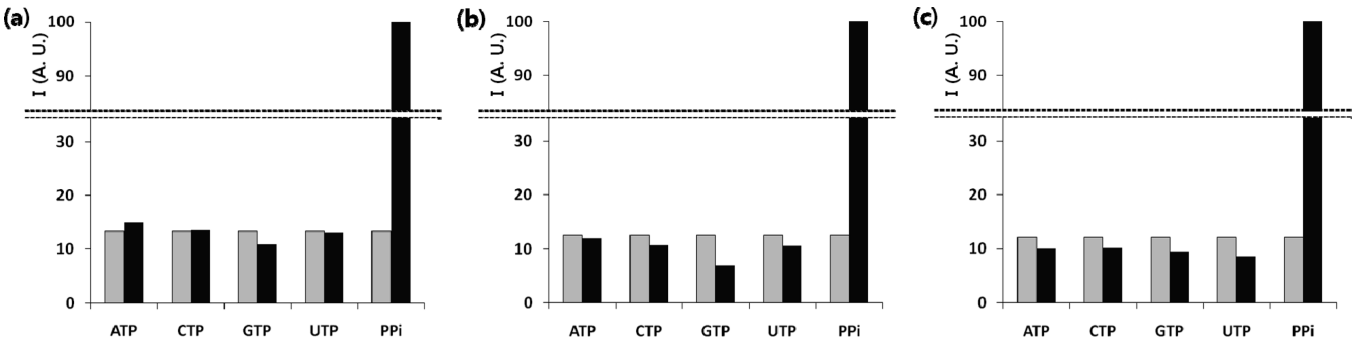


FIGURE 6. Relative fluorescence emission (λ_{ex} = 317 nm) at λ_{max} in CAPS buffer solution (10 mM, pH 10.5) in the absence (gray bar) and presence (black bar) of various anions (10 μM, sodium salts): (a) **1**·2**Zn** (10 μM) and **BA** (10 μM), (b) **1**·2**Zn** (10 μM) and **BA** (20 μM), (c) **1**·2**Zn** (10 μM) and **BA** (40 μM).

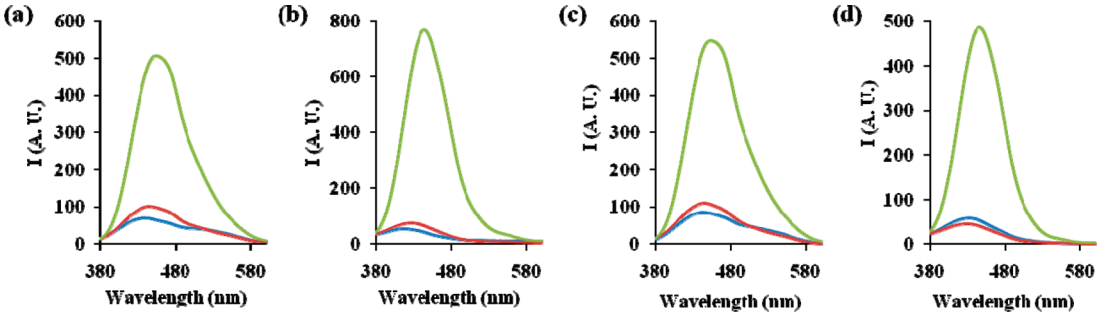


FIGURE 7. Fluorescence emission spectra (λ_{ex} = 317 nm) upon the addition of 10 μM ATPs (red line) and 10 μM PPi (green line) to aqueous solutions (blue line) containing (a) **1**·2**Zn** (10 μM) in CHES (2-(cyclohexylamino)ethanesulfonic acid) buffer (10 mM, pH 9.0); (b) **1**·2**Zn**+**BA** (10 μM + 30 μM) in CHES buffer (10 mM, pH 9.0); (c) **1**·2**Zn** (10 μM) in CAPS buffer (10 mM, pH 10.5); (d) **1**·2**Zn**+**BA** (10 μM + 30 μM) in CAPS buffer (10 mM, pH 10.5).

Subsequent addition of PPi to these NTP-added solutions led to an increase in fluorescence intensity (blue line to black line). It is noteworthy that in the case of **1**·2**Zn**+**BA**, the addition of a small amount (1 μM) of PPi caused a marked change in the fluorescence intensity (1.7 times), as opposed to the case of **1**·2**Zn** (1.3 times). This implies that in the presence of NTPs, **1**·2**Zn**+**BA** is more sensitive to PPi than is **1**·2**Zn**. Thus, by using the **1**·2**Zn**+**BA** sensing system, we can easily distinguish PPi from NTPs.

Effect of BA Concentration on PPi Sensing. Figure 3 shows that the fluorescence emission from **1**·2**Zn** (10 μM) is almost quenched upon the addition of 30 μM **BA**. We believe that optimum selectivity between PPi and NTPs can be achieved if every **1**·2**Zn** molecule binds with **BA**. This is because the coordination between **BA** and **1**·2**Zn** facilitates the reaction between the incoming NTPs and **BA** (Scheme 3).[14]

To confirm our reasoning, the experiments described in Figure 4 were carried out at **BA** concentrations other than 30 μM (Figure 6). As shown in Figures 4 and 6, the selectivity between PPi and NTPs was enhanced with an increase in the amount of **BA** added, but no notable selectivity enhancement could be observed when the BA concentration exceeded 30 μM. This was possibly because an overdose of **BA** could weaken the binding between PPi and **1**·2**Zn**. Therefore, the optimum concentration of **BA** in the current **1**·2**Zn**+**BA** sensing system was found to be 30 μM when 10 μM **1**·2**Zn** was used. The aforementioned concentrations (**1**·2**Zn** = 10 μM, **BA** = 30 μM) of the two chemosensors, **BA** and **1**·2**Zn**, were employed in the following bioanalytical assay.

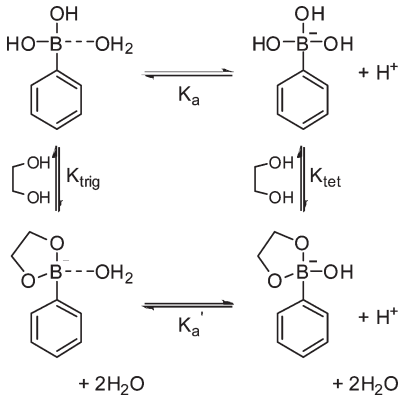
Effect of pH on the PPi Sensing Ability of 1·2Zn+BA. The effect of pH on the efficiency of the **1**·2**Zn**+**BA** sensing system was examined, as shown in Figure 7. The addition of PPi led to a significant increase in the fluorescence intensity, regardless of the pH. However, the addition of ATP brought about a decrease in the fluorescence intensity at pH 10.5 and an increase in the fluorescence intensity at pH 9.0. In other words, the use of **1**·2**Zn**+**BA** enables the efficient distinction between PPi and ATP at pH 10.5.

It has been reported that the selectivity **1**·2**Zn** for PPi in preference to NTPs does not change in the pH range 6.5−10.1.[3b] However, when using the **1**·2**Zn**+**BA** sensing system, the aforementioned selectivity for PPi can be improved by increasing the pH of the medium; this is because the interaction between **BA** and NTPs is strengthened in an alkaline medium. For instance, the association constant between **BA** and D-fructose is reported to be as high as 170 M^{-1} at pH 8.21.[10a] We assume that the binding affinity between **BA** and NTPs is similar to that between **BA** and D-fructose; hence, at micromolar concentrations, the binding between **BA** and NTPs would not be sufficiently strong. The equilibrium of boronic acids such as **BA** is described in Scheme 4.[15] Typically, K_{tet} is higher than K_{trig}, with a difference of up to 5 orders of magnitude.[15] With an increase in the pH, the equilibrium is shifted to the right, and thus, the

(14) (a) Horton, N. C.; Perona, J. J. *Nat. Struct. Biol.* **2001**, *8*, 290–293. (b) Kuo, L. Y.; Piccirilli, J. A. *Biochim. Biophys. Acta* **2001**, *1522*, 158–166.

(15) (a) Bosch, L. I.; Fyles, T. M.; James, T. D. *Tetrahedron* **2004**, *60*, 11175–11190. (b) James, T. D. *Top. Curr. Chem.* **2007**, *277*, 107–152.

SCHEME 4. Equilibrium of a Typical Boronic Acid (from ref 15a)



binding between **BA** and NTPs is more strongly driven by K_{tet} than by K_{trig}. Therefore, an increase in pH is expected to strengthen the binding of **BA** to NTPs, and thus, the fluorescence quenching effect by the **1**·2**Zn**+**BA** sensing system becomes strong upon the addition of NTPs.

RNA Quantification Experiment. We used **1**·2**Zn**+**BA** to quantify the amount of RNA formed during the RNA transcription process; for this purpose, we measured the concentration of PPi released with the transcribed RNA. For reference, we performed a transcription experiment by using a conventional radioisotope ([α-^{32}P]ATP)-based method (Figure 8).[16]

The time course determined by using **1**·2**Zn**+**BA** showed good correlation with that determined by the radioisotope method (Figure 8). The time course determined by using **1**·2**Zn** was reasonably accurate; however, there was a definite difference between the time courses obtained by using **1**·2**Zn**+**BA** and **1**·2**Zn**. In the early stage of the time course experiment performed using **1**·2**Zn**, the amount of transcribed RNA was overestimated. This was because of the fluorescence increase caused by the presence of a large amount of NTPs, which could not be effectively displaced by the small amount of PPi formed in the early stages of RNA transcription. The interaction between NTPs and **1**·2**Zn** could be disturbed because of the continually increasing concentration of PPi during RNA transcription. Therefore, when using **1**·2**Zn**, the amount of transcribed RNA was overestimated in the early transcription stage, when PPi was present in low concentrations.

To confirm the effect of the transcribed RNA on the fluorescence intensity of the sensing system, we extracted the transcribed RNA from the mixture after the transcription process and checked if the addition of the extracted RNA resulted in an increase in the fluorescence intensity of the sensing system (Supporting Information). Surprisingly, the transcribed RNA caused an increase in fluorescence intensity of **1**·2**Zn**, as did the NTPs. The transcribed RNA was also responsible for the overestimation at the early stage of the transcription process. Thus, for accurate RNA quantification by **1**·2**Zn**, the transcribed RNA should be removed by RNase. On the other hand, in the case of the **1**·2**Zn**+**BA** sensing system, the transcribed RNA had no significant effect

(16) Liu, M.; Asanuma, H.; Komiyama, M. *J. Am. Chem. Soc.* **2006**, *128*, 1009–1015.

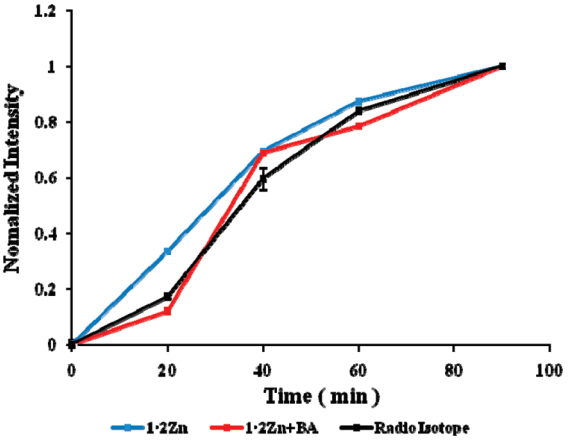


FIGURE 8. Time course of the transcription measured using **1**·2**Zn** (10 μM), **1**·2**Zn**+**BA** (10 μM + 30 μM), and the radioisotope method.

on the fluorescence enhancement. **BA** probably interacted with the ribose moiety of the transcribed RNA, similarly to NTP, and thus, **1**·2**Zn**+**BA** quenched the fluorescence upon binding with the transcribed RNA. Therefore, **1**·2**Zn**+**BA** can be used to directly quantify the amount of transcribed RNA without using RNase.

Conclusion

This study shows a combination of a phenoxo-bridged dinuclear metal complex and boronic acid (**BA**) which can be used for efficient distinction between PPi and NTPs, which are structurally similar and coexist in many biological environments. We have developed a selective PPi-sensing system (**1**·2**Zn**+**BA**), whose fluorescence intensity is selectively increased upon the addition of PPi and decreased upon the addition of NTPs in aqueous solution. **1**·2**Zn**+**BA** never misidentifies NTPs as PPi. The high selectivity of our sensing system for PPi over NTPs can be attributed to the formation of a termolecular complex between **1**·2**Zn**+**BA** and PPi or NTPs. The interaction between **BA** and NTPs is strengthened via the formation of a boronate anion at high pH; this in turn leads to an increase in the concentration of the termolecular complex formed and the efficiency of fluorescence quenching by the bound **BA**. Our sensing system has been successfully used for the accurate quantification of RNAs produced during RNA transcription.

Experimental Section

Instrumentation and Methods. MALDI-TOF mass spectrometry data were obtained by using an Autoflex II TOF/TOF mass spectrometer with α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. Fluorescent spectra were recorded at 25 °C using a Jasco FP-6500 or Molecular Device SpectraMax M2^e.

Synthesis. The two chemosensors (**1**·2**Zn** and **BA**) were synthesized according to previously described methods.[3b,10a] All reagents were purchased from commercial suppliers and used as received.

Transcription by T7 RNA Polymerase (RNAP)[16]. The conditions used for the T7 RNAP reaction were as follows: [T7 RNAP (from a commercial supplier)] = 50 units in 20 μL (corresponding to 0.15 μM); [[α-^{32}P]ATP] = 40 μCi in 20 μL; [NTP] = 0.5 mM in 20 μL, and [each strand of the promoter] = 2.0 μM in 20 μL.

Tris-HCl buffer (42.5 mM, pH 7.9) containing spermidine (2 mM), dithiothreitol (10 mM), 2-mercaptoethanol (1 mM), EDTA (50 μM), $MgCl_2$ (6 mM), and NaCl (0.5 mM) was used. After the addition of T7 RNAP, the reaction mixture was incubated at 37 °C for 1.5 h to effect transcription. During the reaction, a small amount of this mixture was sampled at regular intervals, and the transcription was stopped by heating the mixture to 65 °C. These mixtures were then subjected to electrophoresis on 15% polyacrylamide 7 M urea gel or aqueous solutions of **1**·2**Zn** and **1**·2**Zn**+**BA**. Quantification of the RNA levels separated on the gel was carried out by image densitometry using the Labwork image acquisition and analysis software or by Molecular Device SpectraMax M2^e.

Nontemplate	5′-ATA ATA CGA CTC ACT ATA GGG AGG AAG ATA GAG CA-3′
Template	3′-TAT TAT GCT GAG TGA TAT CCC TCC TTC TAT CTC GT-5′

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Supporting Information Available: Fluorescence emission spectra, MALDI-TOF mass spectra of complexes, and calculations of binding constants. This material is available free of charge via the Internet at http://pubs.acs.org.