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An indicator displacement system for fluorescent detection of phosphate oxyanions under physiological conditions

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Abstract—A fluorogenic chemosensing system is described and shown to selectively detect pyrophosphate under physiological conditions. In the best case, pyrophosphate and hydrogen phosphate are capable of displacing a fluorescent coumarin-derived indicator from a bis Zn^{2+}-dipicolylamine coordination compound with association constants of 10^7 and $10^5\,M^{-1}$, respectively.

The selective recognition of anions with biological importance is a topic of current interest to both chemists and biologists.[1] An increasingly popular strategy for anion recognition is the displacement of a fluorogenic or chromogenic indicator from an indicator–receptor complex by the analyte anion.[2–8] This displacement approach has been used recently in the detection of carbonate,[2] hydrogen sulfate, and hydrogen phosphate,[3] nitrate,[4] phosphate,[5] pyrophosphate,[6] and various halides,[7] as well as the organic anions tartrate and malate.[8] Sensors for pyrophosphate in aqueous solution have been previously reported,[6,9] but most available strategies employ redox-active metal cations as fluorescence quenchers, or use UV chromophores as the indicator. As a part of our ongoing effort to develop sensing systems for cell apoptosis,[10] we needed to develop a fluorescent displacement assay for phosphate oxyanions. The specific goal of this study was to find a suitable fluorescent indicator that could be used in combination with zinc coordination compounds **1**–**4**. The Zn^{2+}-coordination compound **4** has been used as a colorimetric sensor for pyrophosphate in aqueous solution,[5,9a] and we report here that **4** can be used in a fluorescence displacement assay to detect low concentrations of pyrophosphate under physiological conditions (pH 7.4, 5 mM TES, 145 mM NaCl). We have further identified the related Zn^{2+}-coordination compound, **3**, as a receptor with a stronger affinity for pyrophosphate.

Zn^{2+}-coordination compounds **1**–**4** (Fig. 1) were prepared by literature methods[11] and evaluated as receptors for pyrophosphate in a fluorescence displacement assay

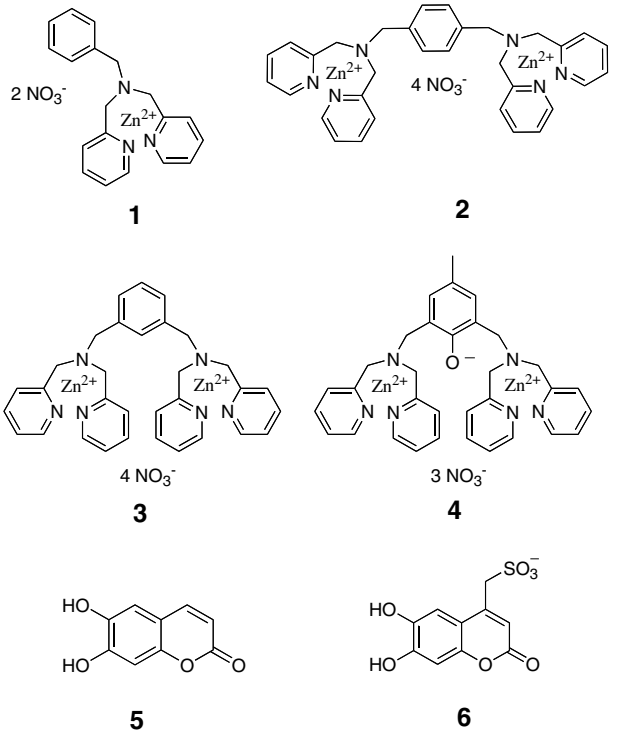

Figure 1. Structure of the Zn^{2+}-DPA coordination compounds **1** through **4** used as receptors, and the fluorescent molecules **5** and **6** used as indicators in the displacement assays.

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using the coumarin-based compounds **5** and **6** as indicators.[12] We first explored the changes in spectral properties of each indicator upon addition of the Zn^{2+}coordination compounds. The requirement to operate under physiological conditions lead us to eliminate the commercially available **5** due to poor solubility in aqueous solution and an unstable fluorescence emission. Sulfonate **6**, which is highly water soluble and exhibits very stable fluorescence emission, was subsequently chosen as the more suitable indicator. Compound **6** was easily prepared in 65% yield by treatment of 6,7-dihydroxy-4-chloromethyl coumarin with sodium sulfite.[13] Initially UV–vis titrations were performed at 25 °C in an aqueous solution (buffered at pH 7.4 using 5 mM TES, 145 mM NaCl) of **6** (100 μM) using **1** through **4** as the titrant. Increasing receptor concentration lead to a decrease in intensity of the indicator absorption band at 375 nm and a simultaneous increase in intensity of the band at 430 nm (Fig. 2).[14]

While this change in the absorption spectrum could be used in a ratiometric assay, a drawback was the relatively high indicator concentration (100 μM) needed to acquire a suitable absorption spectrum. Fluorescence spectroscopy was used in order to evaluate the capacity of small anions to displace the indicator from the receptor at the low concentrations needed for physiological utility. We found that receptors **1** through **4** were each capable of quenching the fluorescence emission of **6** in a concentration dependent fashion (Fig. 3). Jobs plots were prepared for the complex of each receptor and **6**, and a 1:1 binding stoichiometry was found in each case. We determined the association constant for each receptor with **6** by fluorescence titration and subsequent nonlinear curve fitting to a standard 1:1 binding model[15] (Fig. 4). Association constants are given in Table 1.

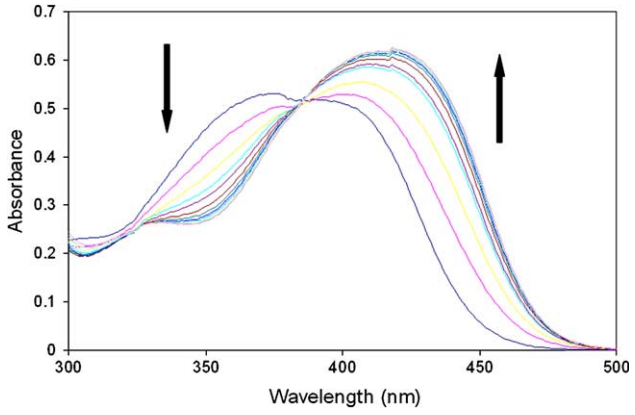

Figure 2. UV–vis spectra of **6** following the addition of increasing amounts of **3**. Compound **3** was titrated into a 100 μM solution of **6** over the concentration range 0–250 μM. All titrations were performed at 25 °C in aqueous solution, pH 7.4, 5 mM TES, 145 mM NaCl.

Anslyn and co-workers have recently shown[8b] that selectivity of a displacement assay for a given analyte can be regulated by variation in the relative concentration of receptor and indicator. In our assay for pyrophosphate, we prepared a 1:1 receptor–indicator complex in aqueous solution buffered at pH 7.4 (5 mM TES, 145 mM NaCl) by mixing equimolar amounts of receptor and indicator (10 μM each). We then titrated this solution with aliquots of the sodium salts of nitrate, phosphate, hydrogen phosphate, pyrophosphate, sulfate, carbonate, and acetate. Only pyrophosphate, and to a lesser extent hydrogen phosphate, were able to displace the indicator from the receptor–indicator complex, as reported by a restoration of fluorescence intensity (Fig. 5). The receptor–indicator complexes of **1** and **2** failed to respond to any of the anions used in this investigation. Pyrophosphate was easily able to displace **6** from the receptor–indicator complexes of **3** and **4**, and addition of hydrogen phosphate resulted in a substantial, though significantly diminished indicator displacement from these same complexes. Fluorescence emission intensities from these titrations ($\lambda_{ex.}$ = 347 nm, $\lambda_{em.}$ = 480 nm) were then analyzed by the standard method for competition assays[16] to determine the association constants presented in Table 2.

Receptors **3** and **4** bound pyrophosphate with nearly two orders of magnitude greater affinity than hydrogen

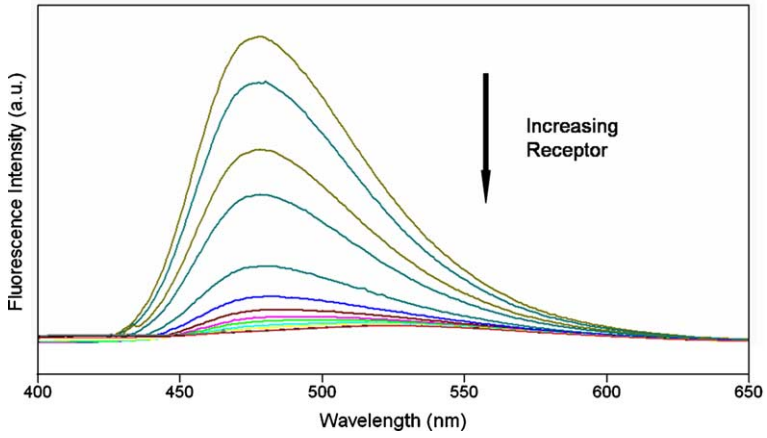

Figure 3. Fluorescence emission spectra of **6** following the addition of increasing amounts of **3**. Compound **3** was titrated into a 10 μM solution of **6** over the concentration range 0–100 μM. Excitation wavelength was 347 nm. All titrations were performed at 25 °C in aqueous solution, pH 7.4, 5 mM TES, 145 mM NaCl.

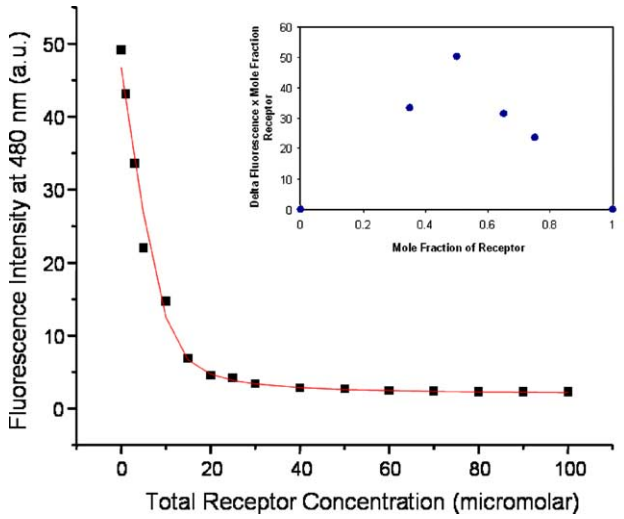

Figure 4. Fluorescence intensity of **6** at 480 nm in the presence of increasing amounts of **3**. Excitation wavelength was 347 nm. Inset: Jobs plot for the receptor–indicator binding of **6** and **3**, clearly indicating a 1:1 stoichiometry. All experiments were performed at 25 °C in aqueous solution, pH 7.4, 5 mM TES, 145 mM NaCl.

Table 1. $K_{association}$ for receptors and indicator ($\times 10^5\,M^{-1}$)[a]

Receptor	Indicator 6
1	1.0 ± 0.1
2	6.0 ± 1.5
3	23.1 ± 16.0
4	1.8 ± 0.3

[a] All titrations were performed at 25 °C in aqueous solutions buffered at pH 7.4 with 5 mM TES, 145 mM NaCl. Each value represents the average of at least three separate experiments.

phosphate. Furthermore, receptor **3** bound both pyrophosphate and hydrogen phosphate more tightly than **4** by nearly an order of magnitude in each case. At pH 7.4 the phenolic proton of **4** will be largely deprotonated, giving **4** an overall charge of +3. The additional +1 charge of **3** may facilitate this increased affinity for the anions by enhancing the electrostatic interaction between the receptor and the analyte. The association constant determined for **4** with hydrogen phosphate, $(11.4 \pm 3.3) \times 10^4\,M^{-1}$, agrees remarkably well with the previously reported[5] value of $(11.2 \pm 0.8) \times 10^4\,M^{-1}$. The association constant for **4** with pyrophosphate, $(6.7 \pm 1.8) \times 10^5\,M^{-1}$, also agrees well with previous reports[9a] after consideration of the greater ionic strength used in the present studies.

Table 2. $K_{association}$ for receptors and anionic species ($\times 10^5\,M^{-1}$)[a]

Receptor	Pyrophosphate	Hydrogen phosphate
1	—	—
2	—	—
3	152 ± 26	7.3 ± 3.7
4	67.2 ± 18.2	1.1 ± 0.3

[a] All titrations were performed at 25 °C in aqueous solutions buffered at pH 7.4 with 5 mM TES, 145 mM NaCl. Each value represents the average of at least three separate experiments.

In summary, a fluorescence-based displacement assay for pyrophosphate has been developed that is fully functional under physiological conditions. The chemosensing ensemble formed from **3** and **6** detects pyrophosphate over hydrogen phosphate with a selectivity difference of nearly two orders of magnitude. Practical applications of this sensing system will be reported in due course.

Acknowledgements

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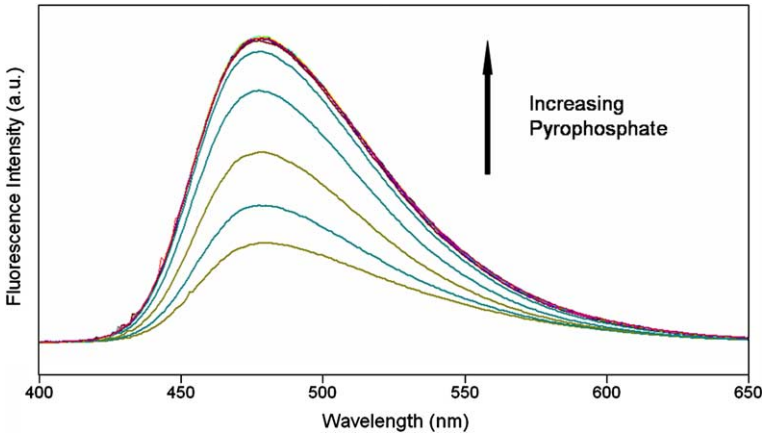

Figure 5. Fluorescence emission spectra of **6** after addition of increasing amounts of sodium pyrophosphate to a 1:1 mixture of **6** and **3** (concentration of the receptor–indicator complex was 10 μM). Excitation wavelength was 347 nm. All experiments were performed at 25 °C in aqueous solution, pH 7.4, 5 mM TES, 145 mM NaCl.

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