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An indicator displacement assay with independent dual wavelength emission

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ABSTRACT

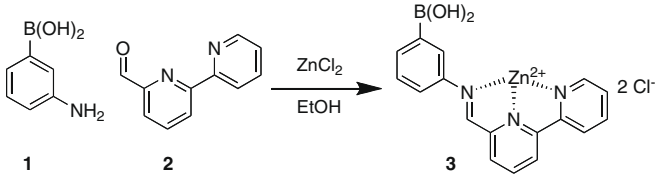
A multifunctional metallo-receptor was designed with both metal and boronic acid binding groups. A sensor ensemble was prepared using the metallo-receptor and the fluorescent dye ARS. The dye produced two distinct fluorescent bands from interaction with the boronic acid and the metal, respectively. Partial displacement of the dye by simple analytes led to different fluorescent signatures than full displacement. This differential response provided easy discrimination of the individual analytes.

Synthetic fluorescent sensors have become very important research tools in many scientific investigations, including biological, medical, and environmental analysis.[1] Utilizing the power of fluorescent imaging technology, fluorescent sensors can provide measurements of specific analytes with high sensitivity, rapid response, and ease of use.[2] Thus much effort has been put into the design and characterization of new sensors for various biologically and chemically important analytes.[3] Although several practically useful sensors for metal ions have been developed,[4] sensors for organic analytes have lagged behind.

The major challenge to be overcome in the design of sensors for organic analytes in a cellular environment is the issue of selectivity, due to the wide variety and similarity of organic biomolecules. Selective receptors are typically approached using multiple recognition elements that match the functional groups of the analyte. A fluorescent response can be built in by a number of methods. One well-established method to construct a sensor is to use an indicator displacement assay (IDA).[5] In this approach, a specific receptor is pre-equilibrated with an indicator (fluorescent reporter), which matches the recognition elements of the receptor. The indicator is chosen such that its optical properties change upon binding and release from the receptor. Upon the addition of the analyte, the indicator is displaced, producing a fluorescence response. The combination of a multifunctional receptor with a fluorescent dye raises the intriguing possibility that a receptor/dye combination could be designed in which the dye could be fully or partially displaced and give different responses. Herein, we report a fluorescent sensor ensemble that gives two independent wavelengths of fluorescence response, depending on the type of analyte that binds.

Recently, we used an indicator displacement method to create a sensor for nucleotides using a combination of metal binding and aromatic tweezer interactions.[6] For the purpose of creating a simple two recognition element receptor, a zinc complex bearing a boronic acid was employed (Scheme 1). Compound **3** was chosen since it was trivially prepared by imine formation between aniline **1** and bipyridyl aldehyde **2**[7] followed by metal coordination.[8] The simplicity of construction allows various derivatives of the receptor to be prepared easily. Anslyn has used a number of boronic acid appended coordination complexes as colorimetric sensors for various analytes.[9] Thus, the baseline expectation for this sensor was that the boronic acid would interact with diols such as those present on sugars and the metal ion would interact with anionic groups such as phosphate. Thus, a phosphosugar was expected to be an appropriate guest for receptor **3**.

To complete the sensor ensemble, the fluorescent dye Alizarin Red S (ARS) was employed (Fig. 1), as it is known to change fluorescence upon interaction with boronic acids.[10] ARS has a very weak fluorescence at 554 nm. It has been shown that upon binding with a boronic acid, the fluorescence of the 554 nm band increases. Displacement of the ARS from the boronic acid by a diol-containing analyte subsequently induces a decrease in fluorescence.[8] Surprisingly, the addition of receptor **3** to ARS leads to an increase in fluorescence in two distinct bands: one at 554 nm and one at 619 nm (Fig. 2d). The band at 554 nm was presumed to arise from the interaction of the ARS with the boronic acid of the receptor based on the literature. This was confirmed by the addition of compound **1** to ARS which gave rise to an increase in the only the 554 nm band (Fig. 2b). We speculated that the band at 619 nm arose from an interaction of the dye with the metal center. Metal complex **4** was prepared to test this hypothesis.[11] Indeed, the addition of



Scheme 1. Preparation of receptor **3**.

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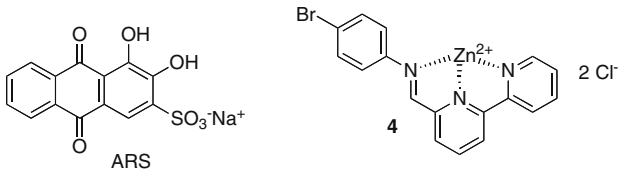

Figure 1. Structures of ARS and control receptor **4**.

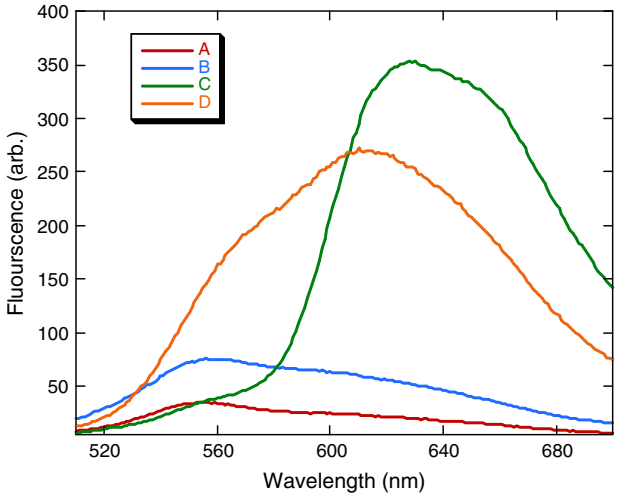

Figure 2. Fluorescence spectrum (λ_{ex} = 495 nm) of ARS (100 μM) in Tris–buffer (25 mM with 120 mM NaCl and 50 mM $Na_2S_2O_3$, pH 7.40). (a) ARS alone; (b) ARS with compound **1** (560 μM); (c) ARS with compound **4** (200 μM); (d) ARS with receptor **3** (200 μM).

compound **4** to the dye gave rise to an increase in only the emission of the 619 nm band (Fig. 2c). Interestingly, Kubo has studied the interactions of metals with ARS–boronic acid complexes in methanol and found that the ARS emission is quenched by most metals including Zn^{2+}.[12] Here, we found that in aqueous solution, the ARS emission was enhanced by the metal at a separate band, in both the boronic acid complex and the free dye.

Titration of ARS with receptor **3** produced a uniform increase in both fluorescence bands, reaching saturation simultaneously (Fig. 3). Fitting the fluorescence change to a single site biding isotherm produced a binding constant of 14,000 M^{-1} regardless of which wavelength was used for the calculation. Furthermore, the binding constant of ARS with compound **1** was only 180 M^{-1} and

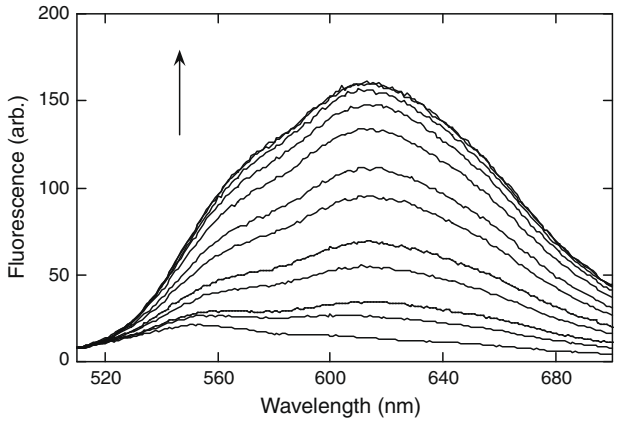

Figure 3. Fluorescent titration (λ_{ex} = 495 nm) of ARS (100 μM) in Tris–buffer (25 mM with 120 mM NaCl and 50 mM $Na_2S_2O_3$, pH 7.40) with compound **3** (0–320 μM).

that of ARS with compound **4** was 8700 M^{-1}. Thus it appears that ARS binds to both functional groups of receptor **3** simultaneously and cooperatively, rather than as a mixture of ARS bound sometimes to the boronic acid and sometimes to the metal ion.

Gratifyingly, the addition of a phosphosugar such as ribose-5-phosphate (Fig. 4a) gave displacement of the dye from the complex with concomitant decrease in both bands of the emission spectrum. Using the typical displacement methods,[13] the binding constant of receptor **3** for ribose-5-phosphate was found to be 780 M^{-1}. Interestingly, the addition of simple sugars such as

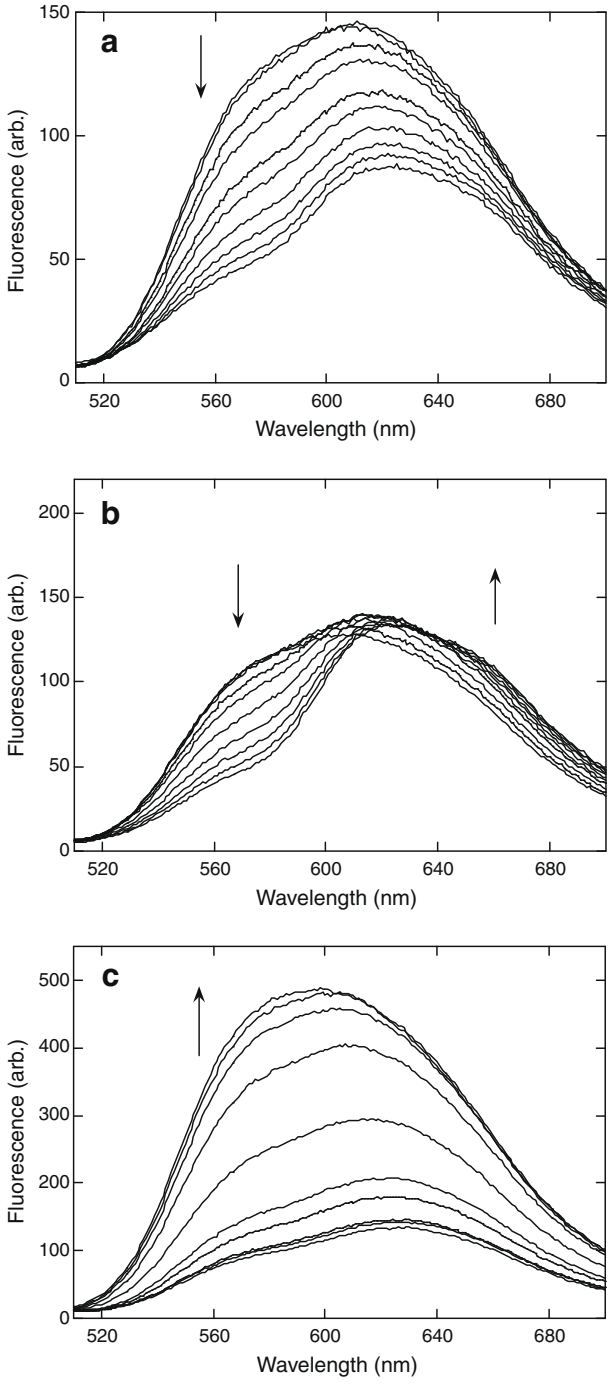

Figure 4. Fluorescent titration (λ_{ex} = 495 nm) of ARS (100 μM) and compound **3** (1 mM) in Tris–buffer (25 mM with 120 mM NaCl and 50 mM $Na_2S_2O_3$, pH 7.40) for the addition of (a) ribose-5-phosphate (0–61 mM) (b) fructose (0–63 mM) (c) pyrophosphate (0–1.0 mM).

fructose (Fig. 4b) and ribose (data not shown) led to a decrease in the 554 nm band and a concomitant small increase in the 619 nm band. In fact, the emission of the ensemble began to greatly resemble that of the ARS–compound **4** complex (Fig. 1c). This result correlates with displacement of the dye from the boronic acid unit, but not the metal. Not only did the simple sugar bind less well than the phosphosugars (estimated K_a = 100 M^{-1} for ribose), the simple sugars gave completely different fluorescent signature. This result also supports our supposition that compound **3** binds the ribose-5-phosphate using both binding groups.

We then decided to test the sensor ensemble against just the phosphate portion of the phosphosugars since it presumably binds to the zinc. We chose pyrophosphate as it is known to bind well to zinc complexes and might serve the purpose of displacing the dye only from the zinc portion of the receptor.[14] The expectation was that upon the addition of pyrophosphate, the emission of only the 619 band would decrease. In the event, titration of the ARS–compound **3** ensemble with pyrophosphate (Fig. 4c) led to a large increase in both emission bands. Phosphate induced the same effect, but with lower affinity (data not shown). We rationalized this result based on similar results by Kubo et al.[12] using simple ARS–boronic acid complexes that demonstrated large increases in emission upon the addition of anionic analytes. The anions presumably form a boronate complex that induces a fluorescence increase. We note that the phosphosugars did not participate in this binding mechanism, presumably because the sugar portion of the analyte displaces the dye. Although the type of fluorescence change was not as anticipated, in fact all three types of analytes gave distinct fluorescence signatures upon binding the sensor ensemble.

Taken together, these data suggest that the sensor ensemble gives the typical dye displacement results for phosphosugar biding. However, because the two emission bands appear to arise independently from interaction of the dye with different functional groups on the receptor, analytes which only interact with only one of the functional groups can result in partial displacement of the dye which in turn produces a unique fluorescent signature. This ability provides a novel method for identifying the type of analyte introduced. In this case, three different analytes could be distinguished: one that produced full displacement, one that produced partial displacement, and one that utilized a separate fluorescence mechanism.

In most ensemble systems, any binding would produce dye displacement and would activate the sensor in the same fashion. In this case, simple sugar binding yields a distinctly different response from the dye than phosphosugars. Furthermore, simple phosphates yield a response that is distinct from either sugars or phosphosugars. Although the mechanism of the response to phosphates is different, these data suggest that this ensemble is unique in its ability to discriminate the binding of the different types of analytes.

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