Boronic acid-linked fluorescent and colorimetric probes for copper ions†

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Received (in Cambridge, UK) 14th August 2008, Accepted 11th September 2008
First published as an Advance Article on the web 14th October 2008
DOI: 10.1039/b814167c

The first examples of boronic acid-linked fluorescent and colorimetric chemosensors for copper ions are reported; the monoboronic acid-conjugated rhodamine probe displays a highly selective fluorescent enhancement with Cu^{2+} among the various metal ions whereas the fluorescence of the bisboronic acid-conjugated fluorescein probe is selectively quenched by Cu^{2+}, probably by way of a PET mechanism.

Metal-selective fluorescent chemosensors serve as useful tools for detection of cellular metal ions and thus have been widely exploited to detect biologically relevant metal ions in cells and/or organisms.[1] Among the various transition metal ions, the copper ion plays a critical role as a catalytic cofactor for a variety of metalloenzymes, including superoxide dismutase, cytochrome *c* oxidase and tyrosinase. However, under overloading conditions, copper exhibits toxicity in that it causes neurodegenerative diseases, probably by its involvement in the production of reactive oxygen species.[2] In this regard, fluorescent probes for copper ions have been extensively explored owing to the biological significance of these metal ions.[3]

Boronic acids are known to have high affinities for substances that contain vicinal diol groups[4] and, consequently, they have been used in fluorescent chemosensors for carbohydrates.[5] In addition, a stilbenyl boronic acid has been utilized as a cofactor in an antibody-based sensor to monitor Hg^{2+}.[6] However, no precedents exist for the use of boronic acid-linked fluorescent chemosensors to detect metal ions directly. Herein, we describe the first example of a boronic acid-linked fluorescent and colorimetric chemosensor for copper ions, in which the unique ring-opening process of rhodamine and fluorescein derivatives is manipulated. In addition, the practical use of the probe is demonstrated by its application to the detection of copper ions in mammalian cells and vertebrate organisms.

Synthesis of the rhodamine B boronic acid **1** was accomplished by coupling rhodamine hydrazide[7] with 2-formylphenylboronic acid (Fig. 1).[8] Fluorescein bisboronic acid **2** was prepared by condensing 2,7-dichlorofluorescein and 2-(*N*-methylaminomethyl)phenylboronic acid through Mannich reaction.[8] In addition, rhodamine derivative **3**[3d] and fluorescein derivative **4**[10] which lack boronic acid as well as simple boronic acid derivative **5** without other metal recognition sites were used as controls for elucidating the importance of the boronic acid moieties.

The rhodamine boronic acid **1** forms a colorless solution in 20 mM HEPES (0.5% CH_3CN) at pH 7.4, showing that it exists in the spirolactam form predominantly.[3a,d,11] Upon addition of Cu^{2+} to this solution, a pink color develops and the resulting species exhibits strong orange fluorescence. The absorption (λ_{max} = 556 nm) and emission (λ_{max} = 572 nm) changes are associated with Cu^{2+}-induced spirolactam ring opening that forms the Cu^{2+}–**1** complex (Fig. 2a). When an excess of the copper ion chelator (cyclen) is added to the solution containing the Cu^{2+}–**1** complex, the pink color and yellow fluorescence disappear, and **1** is regenerated, which was confirmed by HPLC analysis of the cyclen-treated Cu^{2+}–**1** complex. Thus, Cu^{2+}-induced spirolactam ring opening is a reversible process. A Job plot shows that binding of **1** to Cu^{2+} has 1 : 1 stoichiometry. The association constant of **1** for Cu^{2+} was determined to be 2.8×10^3 M^{-1} based on the results of a fluorescent titration experiment.[12]

Importantly, other metal ions, such as Ag^+, Ca^{2+}, Co^{2+}, Cd^{2+}, Cs^+, Hg^{2+}, K^+, Li^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^{2+}, Rb^+, Sr^{2+} and Zn^{2+}, do not promote color and fluorescence changes in **1** (Fig. 2b). Metal ion competition experiments show that binding of **1** to copper ions is not affected by other metal ions including 100 mM of Na^+ or K^+.[8] It was found that results obtained from addition of the interfering metal ions followed by the addition of the Cu^{2+} to **1** were similar to those obtained in a reverse way. These results indicate that **1** responds to copper ions with a high selectivity. In addition, **1**

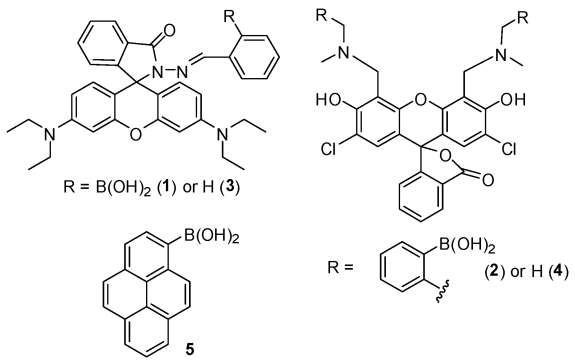

Fig. 1 Structures of boronic acid-conjugated chemosensors **1** and **2** and control compounds **3**–**5**.

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† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b814167c

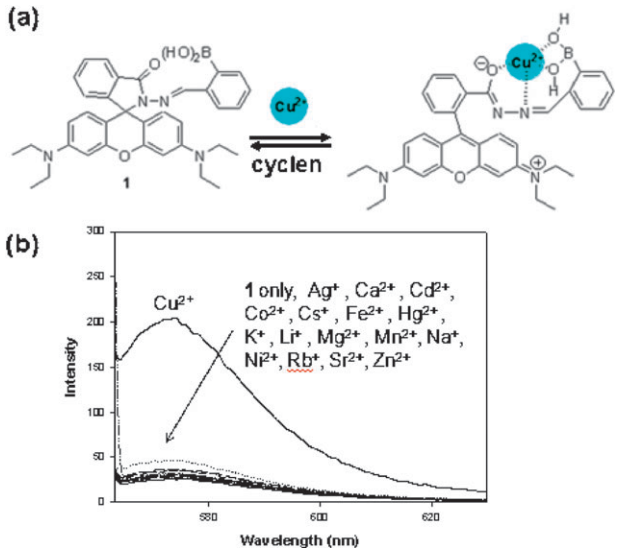

Fig. 2 (a) A proposed binding mode of **1** to copper ions. (b) Fluorescence spectra of **1** (3 μM) upon addition of various metal ions (300 μM) in 20 mM HEPES (0.5% CH_3CN) at pH 7.4 (excitation at 556 nm).

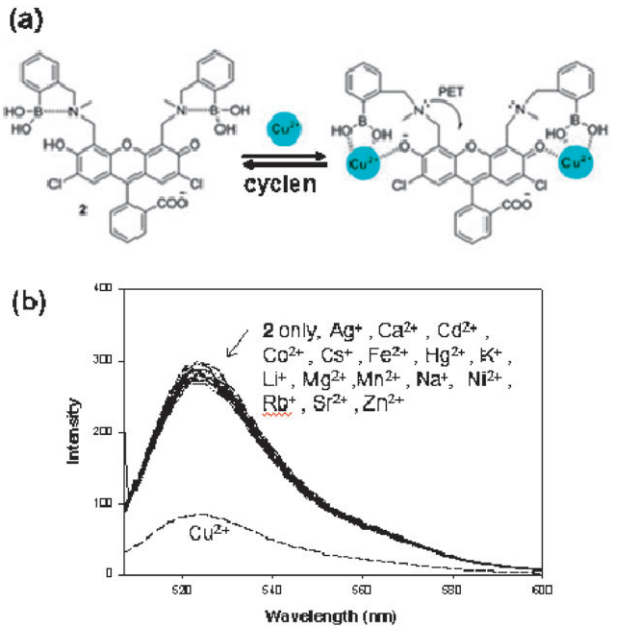

Fig. 3 (a) A proposed binding mode of **2** to copper ions. (b) Fluorescence spectra of **2** (1 μM) upon addition of various metal ions (100 μM) in 20 mM HEPES (0.5% CH_3CN) at pH 7.4 (excitation at 504 nm).

did not display any significant fluorescent change upon addition of vicinal diol-containing carbohydrates such as glucose and fructose (1 mM, respectively).

In order to probe the role played by the boronic acid group in copper ion binding, the rhodamine derivative **3** was treated with Cu^{2+} in HEPES (0.5% CH_3CN) at pH 7.4. No changes in the fluorescence properties of **3** were detected, suggesting the importance of the boronic acid moiety in **1** for binding to copper ions. As another control, the simple boronic acid derivative **5** without other metal recognition sites was also examined with Cu^{2+} under the same conditions. Pyrene boronic acid (**5**) displayed a unique excimer peak at 455 nm in acetonitrile upon addition of Cu^{2+}, indicating the interaction between a boronic acid group and Cu^{2+}.[8] A Job plot indicates that binding of **5** to Cu^{2+} has 2 : 1 stoichiometry.[8] Therefore, it is likely that the excimer is formed by the stacking between pyrene rings through interaction between a boronic acid moiety and Cu^{2+}.[12] Based on the fluorescent and colorimetric change of **1** as well as control experiments, it is proposed that binding of Cu^{2+} to **1** can induce the ring-opening process of spirolactam as shown in Fig. 2a. Czarnik *et al.* reported the first example of a rhodamine B ring opening process for the detection of Cu^{2+}, in which rhodamine B hydrazine was utilized as a chemodosimeter for Cu^{2+}.[7] Unlike this example, our process was reversible as described earlier.

The bisboronic acid-linked chemosensor **2** displays a completely different type of fluorescence response upon interaction with copper ions. In the absence of copper ions, the solution of **2** in 20 mM HEPES (0.5% CH_3CN) at pH 7.4 is a light yellow color and it exhibits strong yellow fluorescence. The addition of Cu^{2+} to this solution causes a color change from light yellow to pink and a concomitant, large intensity reduction and a small blue-shift (~9 nm) of the fluorescence (Fig. 3). The association constant of **2** for Cu^{2+} was determined to be 8.4×10^6 M^{-2} by using fluorescent titration.[12] Finally, chemosensor **2** shows a high selectivity for Cu^{2+} over other metal ions (Fig. 3b). Metal ion competition experiments show that binding of **2** to copper ions is not affected by other metal ions including 100 mM of Na^+ or K^+.[8]

The boron–nitrogen interaction, which is well documented in preceding reports, can block the photo-induced electron transfer (PET) mechanism from benzylic nitrogen.[5] This phenomenon can be applied to explain that chemosensor **2** displays a relatively strong fluorescence. Upon addition of Cu^{2+}, these ions are complexed with boronic acid and phenol OH's as shown in Fig. 3a. This process can liberate a tertiary amine moiety, which can revive the PET process and induce fluorescent quenching. The color change also supports the Cu^{2+} binding to phenolic oxygen, which leads to the formation of phenolate.[13] The effect of the boronic acid in **2** on copper binding was examined by incubating **4** with Cu^{2+} in HEPES (0.5% CH_3CN) at pH 7.4. Fluorescence changes of **4** induced by copper ions were not observed, indicating the importance of the boronic acid moiety in **2** to the binding to copper ions.

Studies of the pH-dependent response of **1** to copper ions were also carried out in the pH range 5.0–9.0. According to these experiments, **1** responds to copper ions at pH 5.0–9.0 in a similar way.[8] The relative quantum yields of **1** were calculated as 0.07 (in the absence of Cu^{2+}) and 0.45 [in the presence of Cu^{2+} (100 equiv.)].[9] Those for **2** were 0.75 (in the absence of Cu^{2+}) and 0.23 [in the presence of Cu^{2+} (50 equiv.)], respectively.[9]

The ability of the boronic acid-linked rhodamine probe **1** to detect copper ions in mammalian cells and living organisms was examined. Murine P19 embryonic carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and then treated with 300 μM $Cu(ClO_4)_2$ for 30 min. The cell cultures were washed with PBS to remove the remaining copper ions and incubated with 50 μM of **1** for 30 min. The results of

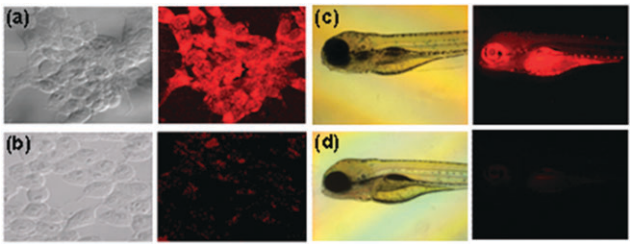

Fig. 4 Cells and organisms incubated with 50 μM **1** (0.5% CH_3CN) and 300 μM $Cu(ClO_4)_2$. Left: Images of P19 cells treated with **1** in the (a) presence and (b) absence of external copper ions. Right: Five-day-old zebrafish treated with **1** in the (c) presence and (d) absence of external copper ions.

fluorescence microscopic analyses of treated cells show that **1** passes through cell membranes and displays a strong fluorescence response to intracellular copper ions (Fig. 4a). In contrast, cells that are treated with only **1** in the absence of external copper ions display very weak fluorescence (Fig. 4b).

Whole organism experiments were also carried out to examine if the probe **1** can be used to detect copper ions in living organisms. A five-day-old zebrafish was treated with 300 μM $Cu(ClO_4)_2$ in E3 embryo media for 30 min at 28 °C and washed with PBS to remove the remaining copper ions.[11c,d] The treated zebrafish was then incubated in a solution containing 50 μM **1** for 30 min. Fluorescence microscopic images show that copper ions in zebrafish are fluorescently detected by **1** (Fig. 4c). However, a zebrafish treated with only **1** in the absence of external copper ions shows a very weak fluorescence signal (Fig. 4d). Fluorescence images with different concentrations of copper ions are also provided in the supporting information.†

In conclusion, the above studies have led to the development of the first boronic acid-linked fluorescent and colorimetric probes **1** and **2**, which selectively respond to copper ions. The chemosensors display opposite fluorescent changes upon complexation with Cu^{2+}. While the fluorescence of the monoboronic acid-linked rhodamine derivative **1** is greatly enhanced in the presence of Cu^{2+}, that of the bisboronic acid-conjugated fluorescein probe **2** is selectively quenched by Cu^{2+}. Both chemosensors also facilitate "naked-eye" detection of Cu^{2+}. In addition, we have demonstrated that the rhodamine probe **1** can be used to detect copper ions in mammalian cells and organisms. We believe that these observations regarding the interaction between boronic acid and Cu^{2+} should serve as the foundation for new strategies to design chemosensors.

This work was supported by grants from the NRL program (R04-2007-000-2007-0 and R0A-2005-000-10027-0) and the SRC program of KOSEF/MOST (R11-2005-008 and R11-2000-070). S.-K. K. thanks the Seoul Science Fellowship program. S.-K. K., S. K. K., H. N. L and J.-M. K. also thank the BK21 program (KRF).

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