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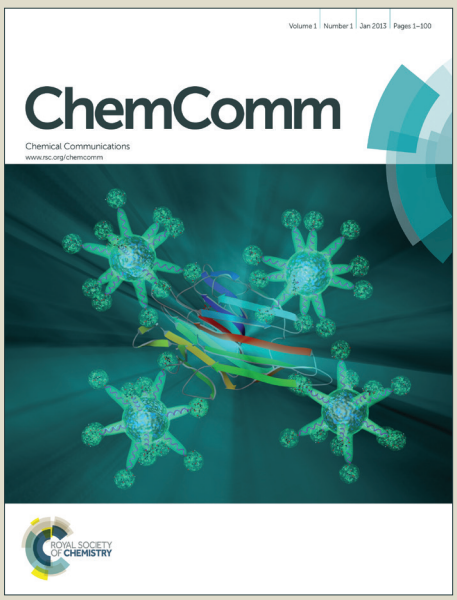

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Highly selective fluorescence imaging of zinc distribution in HeLa-cell and *Arabidopsis* using a naphthalene-based fluorescent probe

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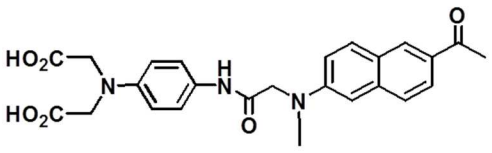
2-(N,N-dimethyamino)naphthalene-based probe 1 was found to dramatic enhancement fluorescence upon addition of Zn^{2+}, but not with any other metal ions. Probe 1 as chemoprobe displayed high-resolution fluorescence imaging for zinc ion in the HeLa-cell and Arabidopsis.

Zinc ion is the second-most abundant metal ion in the human brain and is an active component in growth and reproduction of all organism.[1,2] It plays a key function as a cofactor and a structural elemental in macromolecules and is required for the activity of an estimated 300 proteins including transcription factors regulating gene expression and enzymes involved in metabolism and detoxification of reaction oxygen species (ROS) in plant mitochondria.[3,4] Furthermore, zinc plays an important role in many biochemical reactions within the plants.[5-12] Plants such as maize and sorghum and sugarcane show reduced photosynthetic carbon metabolism due to zinc deficiency. Zinc modifies and/or regulates the activity of carbonic anhydrase, an enzyme that regulates the conversion of carbon dioxide to reactive bicarbonate species for fixation as carbohydrates in these plants.[5-12] Zinc is also a part of several other enzymes such as superoxide dismutase and catalase, which prevent oxidative stress in plant cells.[8]

Although Zn^{2+} has many important cellular roles,[13] its physiological significance is little understood. Therefore, several chemical tools for measuring Zn^{2+} in biological samples have been developed. A variety of fluorescent probes for Zn^{2+}, based on benzothiazole, fluorescein, quinolone, and protein, have been reported.[14-17] Some of these probes can be used under physiological conditions, but they suffer from problems such as inadequate selectivity, insufficient sensitivity, dependence of fluorescence upon the dye concentration, and so forth. In addition, these fluorescent probes have been used to investigate imaging techniques at the cellular level. However, these probes have not yet been tested for their application in plant tissues. Thus, the development of a selective fluorescent probe for Zn^{2+} in plants is a worthwhile goal.



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With these ideas in mind, we have designed a new fluorescent probe for Zn^{2+} derived from 2-(*N,N*-dimethylamino)naphthalene as the receptor, which is soluble in aqueous solution. We report here highly selective fluorescent probe **1** for Zn^{2+} which functions well in the presence of other metal ions such as Na^{+}, Pb^{2+}, Mg^{2+}, Hg^{2+}, Ag^{+}, Cu^{2+}, Co^{2+} and Cd^{2+} in aqueous solution. In practical applications, we also show highly selective fluorescent images of **1** for Zn^{2+} in HeLa cells and in plant tissues.

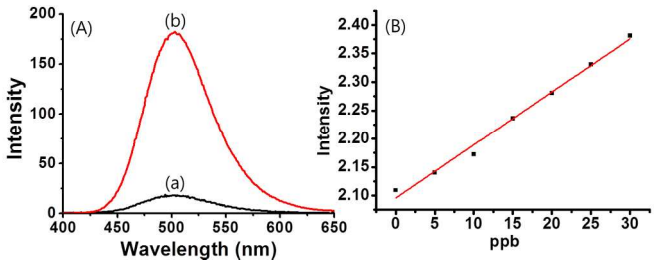

Fig. 1 (A) Fluorescence spectra of (a) free ligand **1** (1.0 x10^{-5} M) and (b) in the present of $Zn(NO_3)_2$ (1.0 ×10^{-4} M) in aqueous solution at pH=7. (B) Plot of fluorescence intensities against concentrations of Zn^{2+} at 500 nm.

We conducted an investigation of the recognition capabilities of **1** for Zn^{2+} in a series of UV-Vis and fluorescence spectroscopy studies. The first observation of the zinc ion detection capability revealed a correlation between the absorption properties of **1** and the concentration of Zn^{2+} in aqueous solution (Fig. S1). The absorbance of **1** (ε= 9.30 x 10^4 M^{-1} cm^{-1}) at ~ 370 nm increased upon addition of Zn^{2+}. The fluorescence emission of **1** (excitation at 370 nm) at 500 nm increased significantly upon addition of Zn^{2+}, with a fluorescence enhancement of up to ~10-fold (Φ= 0.089) with the addition of 10 equivalent of Zn^{2+} (Fig. 1A). This "turn-on" mechanism can be attributed to the blocking of the photoinduced

electron transfer process[18] and the decrease in the electron-donating ability of the amino group by the complexation of **1** with Zn^{2+}. The addition of Zn^{2+} resulted in a linear increase in the fluorescence intensities of **1** (Figs. 1B and S2), indicating that Zn^{2+} was quantitatively bound to **1**. Probe **1** also showed excellent sensitivity with detection of 3.2 ppb of Zn^{2+}, a level which is sensitive enough for practical applications in measurements of Zn^{2+} in biological fluids. In addition, the Job plot using the absorption changes indicated 1:1 binding for **1** with Zn^{2+} (Fig. S3). We further investigated the interaction between **1** and Zn^{2+} by using ESI-mass spectroscopy (Fig. S4). The ESI-mass spectrum of 1 upon addition of Zn^{2+} corresponded to the $[\mathbf{1}^{-}+Zn^{2+}-H_2O]^{+}$ at m/z ~ 545.84 indicating that the binding between **1** and Zn^{2+} led to a 1:1 stoichiometric ratio. The association constant (K_a) of **1** with Zn^{2+} was calculated for 1:1 stoichiometry on the basis of a Benesie-Hildebrand plot,[19] and it was calculated to be 7.51 x 10^4 M^{-1} (Fig. S5). In general, Zn^{2+} acts as soft acid by HSAB theory.[20-21] Therefore, Zn^{2+} is strongly bound to the nitrogen and oxygen atoms of *N,N*-bis(acetic acid)aniline moiety of **1** (Fig. S6).

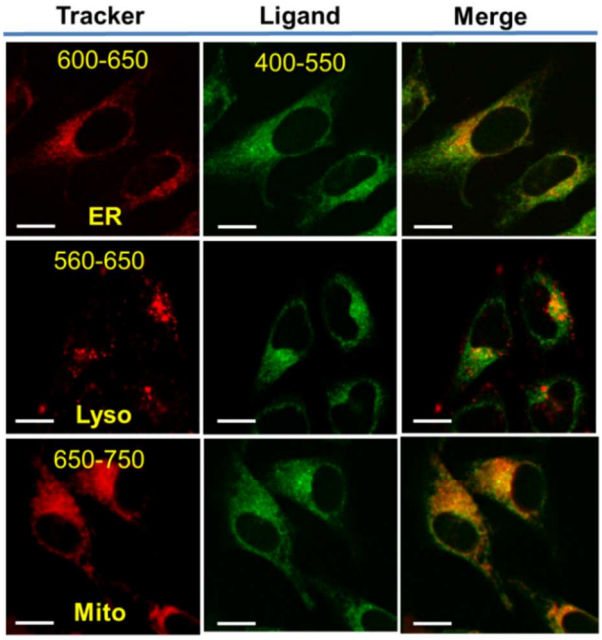

Fig. 2 Confocal fluorescent images of HeLa cell incubated with 5 x 10^{-6} M of **1** with $Zn(NO_3)_2$ (1.0 ×10^{-4} M) for 30 min, λ_{ex} = 740 nm. The excitation wavelengths were 740 nm, 543 nm and 633 nm for ER tracker Red, Lyso Tracker Red DND-99 and Mito Tracker Deep Red FM, respectively.

We also investigated the binding ability of **1** to serve as an ion-selective fluorogenic probe upon the binding of other metal ions including Na^{+}, Pb^{2+}, Mg^{2+}, Hg^{2+}, Ag^{+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Fe^{3+} and Cd^{2+}. However, no significant spectral changes were observed upon addition of any of these metal ions (Fig. S7), indicating that complex **1** is a highly selective chemoprobe for the detection of Zn^{2+}.

To determine if the specificity of detection for Zn^{2+} by **1** was still functional in the presence of an excess of other metal ions, we carried out competition experiments (Fig. S8). The addition of various metal ions (100 equivalents) in aqueous solution at pH 7 to the solution of **1** containing 10 equivalents of Zn^{2+} did not induce any significant changes. Hence, **1** may serve as a selective chemoprobe for Zn^{2+} even in the presence of other relevant metal ions. Comparison of this response across a large pH range revealed that this characteristic fluorescence enhancement by Zn^{2+} exposure was unaffected between pH 3-11 (Fig. S9), a sign of the strong complex formation between the receptor motif of **1** and Zn^{2+}.

We performed two types of cell imaging for Zn^{2+} by using **1** as a chemoprobe for practical applications. In Fig. S10, HeLa cells that were incubated with 5 x 10^{-6} M of **1** for 30 min showed fluorescence emission through a two-photon absorption by its 2-(*N,N*-dimethylamino)naphthalene moiety at λ_{ex} = 740 nm. However, after pre-incubation with pyrithion-Zn^{2+} 1:1 complex (2 x 10^{-5} M) for 40 min, the fluorescence intensity was dramatically increased, which was likely due to Zn^{2+} chelation of **1**, in a similar manner to that in Fig. 1. This result indicates that compound **1** can selectively sense Zn^{2+} ions in a cell. The conclusion was confirmed by the observation that addition of 0.1 mM TPEN (*N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine), a Zn^{2+} ion-selective chelator,[22] to the Zn^{2+}-treated cells reduced the fluorescence intensity.

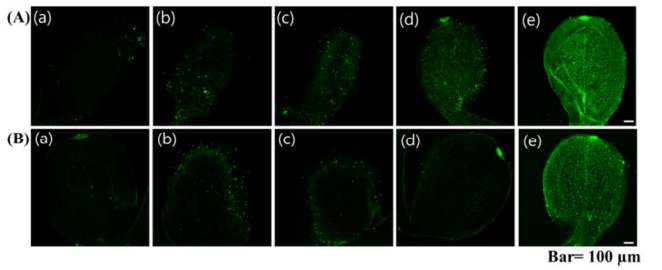

Fig. 3 (A) Fluorescence images of plants treated with different concentration of $ZnSO_4$. Five-day-old seedlings pre-treated with different concentration of $ZnSO_4$ (a) Mock (b) 50 μM (c) 100 μM (d) 500 μM and (e) 1mM for 6 h were incubated with probe **1** (100 μM). (B) The specificity of probe **1** (100 μM) in plant. Six hours (a) before and (b~e) after treatment of (a) Mock (b) $AgNO_3$ (1 mM), (c) $CuSO_4$ (1 mM), (d) $MnSO_4$ (1 mM) and (e) $ZnSO_4$ (1 mM), seedlings were treated with probe **1** (100 μM) for 5 min. Fluorescence was visualized using confocal laser scanning microscope.

To identify the cellular location of Zn^{2+} accumulation, a series of colocalization experiments with organelle selective trackers was performed for the fluorescence image of compound **1** in HeLa cells (Fig. 2). In the panels, the image of the mitochondrial tracker mostly overlapped that of **1** where Pearson's correlation coefficient was 0.85, significantly higher than obtained for other trackers. The mitochondrial targeting of **1** is very interesting considering that this probe has two carboxylate groups, in contrast with general motifs for other molecules that track mitochondria which typically have large hydrophobic cation structures. In this study, we think that probe **1** spreads over the whole cell without preference to mitochondria and its emission increases when it forms a complex with Zn^{2+} ion, Likewise, the apparent mitochondrial preference is due to the localization of Zn^{2+} in mitochondria.

We also investigated the practical applicability of **1** as a Zn^{2+} probe to function within living systems like plants. Five-day-old *Arabidopsis* seedlings treated with different concentrations of $ZnSO_4$ for 6 h were incubated with 100 μM of fluorescent probe **1**, and the fluorescence signal was observed using a confocal laser scanning microscope. As shown in Fig. 3, **1** gave a sufficiently vigorous change in emission intensity under Zn^{2+} pre-treatment conditions, and fluorescence intensity increased in a dose-dependent manner with increasing Zn^{2+} concentrations in *Arabidopsis* seedlings. Although a low background signal was observed in mock-treated seedlings due to a basal signal from probe **1**, the increasing signal with Zn^{2+} addition indicated that autofluorescence from a variety of plant biomolecules including chlorophyll, carotene, and phenolic compounds did not appear to have any effect on probe **1**-mediated fluorescence emission in plants. The high level of fluorescence signal was observed in vascular tissue and epidermal cells,

suggesting that Zn accumulates in both cells of the cotyledons under the Zn^{2+} treatment condition (Fig. 3A). The loading of Zn^{2+} into the apoplastic xylem was required for translocation of Zn^{2+} from the root to the shoot and leaf.[23] In addition, trichomes and epidermal cells are known as a site which accumulate the highest concentrations of Zn^{2+}, although Zn^{2+} distribution between cells is not fully understood.[24]Therefore, these results indicate that the high level of probe **1**-mediated fluorescence emission in vascular tissue and epidermal cells is due to the Zn^{2+} movement to the major storage site. Furthermore, the fluorescence intensity from probe **1** remained constant over 2 hours (Fig. S11), indicating that probe **1** formed stable complex with Zn^{2+} under physiological conditions.

In vitro tests suggested that probe **1** displayed high specificity for binding to Zn^{2+} in preference to other metal ions. In order to confirm the specificity of probe **1**, probe **1**-mediated fluorescence emission from Zn^{2+}-treated seedlings was compared with seedlings pre-treated with different metal cations. As expected, probe **1** was not influenced by other metal ions, such as Ag^{+}, Cu^{2+} and Mn^{2+}, which exist in nature in concentrations as high as 1 mM (Fig. 3B). Taken together, the stability and specificity of probe **1** in physiological conditions indicate its great potential for biological applications.

In conclusion, we have developed a highly selective "turn-on" fluorescent probe for Zn^{2+} in the presence of other metal ions, with 3.2 ppb sensitivity and pH insensitivity in the biologically relevant range. Zn^{2+} imaging by observing the ion interaction with the fluorescent probe **1** was demonstrated using probe **1** in HeLa-cell and plants as practical applications. In particular, probe **1** exhibited a strong fluorescence imaging for Zn^{2+} accumulated in the mitochondrial part of HeLa cells. Application of fluorescent probe **1** for plant cell imaging suggested that Zn^{2+} absorbed from growth media might be sequestered in the vacuole in *Arabidopsis*. These results highlight the capability of **1** for visualization of Zn^{2+} accumulated in plants at the organelle level. Thus, the use of **1** enables micrometer-level analysis of Zn^{2+}, and we anticipate that this capability will soon be extended to the nanoscale.

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Notes and references

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1 C. J. Frederickson, J. Y. Koh and A. I. Bush, *Nat. Rev. Neurosci*., 2005, **6**, 449–462.
2 A. I. Bush, W. H. Pettingell, G. Multhaup, M. d. Paradis, J.-P. Vonsattel, J. F. Gusella, K. Beyreuther, C. L. Masters and R. E. Tanzi, *Science*, 1994, **265**, 1464–1467.
3 W. F. Yuen, K. P. Fung, C. Y. Lee, Y. M. Choy, S. K. Kong, S. Ko and T. T. Kwok, *Life Sci.,* 2000*,* **67**, 725–732.
4 Z. Wang, F. Cai, X. Chen, M. Luo, L. Hu and Y. Lu, *PLOS ONE, 2013,* **8**, 75044.
5 S. Sinha, G. Dey, S. Kumar, J. Mathew, T. Mukherjee, S. Mukherjee and S. Ghosh, *ACS Appl. Mater. Interfaces,* 2013, **5**, 11730−11740.
6 M. P. Cuajungco, G. J. Lees, *Neurobiol. Dis.,* 1997, **4**, 137−169.
7 D. W. Choi, J. Y. Koh, *Annu. Rev. Neurosci*., 1998, **21**, 347−375.
8 W. Maret, C. Jacob, B. L. Vallee, E. H. Fischer, *Proc. Natl. Acad.Sci. U.S.A.,* 1999, **96**, 1936−1940.
9 P. D. Zalewski, I. J. Forbes, W. H. Betts, *Biochem. J.,* 1993, **296**, 403−0.
10 K. H. Falchuk, *Mol. Cell. Biochem.,* 1998, **188**, 41−48.
11 W. Chyan, D. Y. Zhang, S. J. Lippard and R. J. Radford, *PNAS*, 2014, **111**, 143−148.
12 P. J. Dittmer, J. G. Miranda, J. A. Gorski and A. E. Palmer, *J. Biol. Chem.,* 2009*,* **284***,* 16289−16297.
13 E. M. Nolan and S. J. Lippard, *Acc. Chem. Res*. 2009, **42**, 193.
14 K. Komatsu, Y. Urano , H. Kojima and Tetsuo Nagano, *J. Am. Chem. Soc.*, 2007, **129** , 13447–13454.
15 S.C. Burdette, G. K. Walkup, B. Spingler, R. Y. Tsien and S. J. Lippard, *J. Am. Chem. Soc.*, 2001, **123**, 7831–7841.
16 M. Akula, P. Z. E.-Khoury, A. Nag and A. Bhattacharya, *RSC Adv.*, 2014,**4**, 25605-25608.
17 C. E. Outten and T. V. O'. Halloran, *Science,* 2001, **292**, 2488-2492.
18 H. Y. Lee, D. R. Bae, J. C. Park, H. Song, W. S. Han and J. H. Jung, *Angew. Chem. Int. Ed.*, 2009, **48**, 1239 –1243.
19 K. A. Conners, Binding Constants, The Measurement of Molecular Complex Stability; Wiley: New York, 1987.
20 R. G. Pearson, *J. Am. Chem. Soc.*, 1963, **85**, 3533.
21 G. Rayner-Canham, T. Overton, in *Descriptive Inorganic Chemistry*, W. H. Freeman, 4th edn., 2006.
22 R. J. Cousins, R. K. Blanchard, M. P. Popp, L. Liu, J. Cao, J. B. Moore and C. L. Green, *PNAS*., 2003, **100**, 6952-6957.
23 M. Hanikenne, I. N. Talke, M. J. Haydon, C. Lanz, A. Nolte, P. Motte, J. Kroymann, D. Weigel and U. Krämer, *Nature,* 2008, **453**, 391-395.
24 S. A. Sinclair, U. Krämer U, *Biochim Biophys Acta.,* 2012, **1823**, 1553-1567.

Graphical abstract