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Design, synthesis, and evaluation of peptidyl fluorescent probe for Zn^{2+} in aqueous solution

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Abstract—A new fluorescent peptide probe for the detection of Zn^{2+} was synthesized on the basis of zinc binding ligands in zinc enzymes. The peptide that has a unique amino acid sequence displayed a great selectivity for Zn^{2+} in the presence of several transition metal ions in aqueous solution. The reversibility, binding stoichiometry, binding affinity, and pH sensitivity of the sensor were studied. Further, on-bead application of the peptide as chemosensors was demonstrated.

The monitoring of transition metal ions with a selective chemical probe is important in environmental and biological fields. Zn^{2+} is the second most abundant transition metal in the human body, and is essential for biological functions, such as gene expression, apoptosis, enzyme regulation, and neurotransmission.[1] For example, Zn^{2+} is also known to be responsible for the formation of amyloid plaques during the beginning of Alzheimer's disease.[2] The total concentration of Zn^{2+} in different cells varies from the nanomolar range up to about 0.3 mM.[3] Thus, optimized chemical probes are required to monitor zinc concentration ranging from nanomolar to micromolar. Although several fluorescence-based chemical and peptide probes for Zn^{2+} have been developed, most of them suffered limitations due to tight binding affinity (subnanomolar affinity), interference of other metal ions, susceptibility to pH, difficult synthesis, and poor solubility in physiological buffer solution.[4] It is therefore necessary to develop new probes that have micromolar affinity for Zn^{2+} with a high selectivity.

In the present study, we focused on the development of short fluorescence peptide sensors for Zn^{2+} for the following reasons. Peptide probes consisting of natural amino acid can be easily synthesized in solid phase synthesis and can be facilely conjugated to solid support in the device. The sensitivity and selectivity of peptide probes can be optimized by further tuning of the amino acid sequences. As it is difficult for peptide sensors to penetrate into cells due to their hydrophilic property, they are used for monitoring extracellular or environmental metal ions. However, as incorporation of additional functionality is also feasible via amide bond formation, a cellular internalization sequence can be appended to transport the probes into cells in future applications.[5]

Several fluorescence-based peptide sensors have been developed for Zn^{2+}. Zinc peptide sensors were synthesized based on zinc finger domains.[4i] These peptides, consisting of 24 amino acids, suffered limitations with subnanomolar affinity, difficult synthesis due to their size, and the susceptibility of the Cys residues to air oxidation. Peptide sensors containing an unnatural amino acid and β turn sequence have been synthesized.[4j] These peptides form 1:1 or 1:2 complexations with Zn^{2+} and their affinities ranged from nanomolar to 1 μM. However, the peptide probes with micromolar binding affinity for Zn^{2+} bind tightly with Mg^{2+} and Cd^{2+} and

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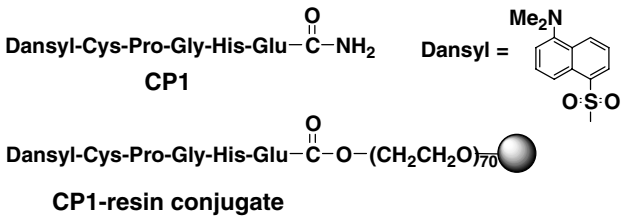
require the difficult synthesis. Thus, the design and synthesis of selective peptide probes with micromolar binding affinity for Zn^{2+} remains a significant challenge.

Considering the drawbacks of the previous peptide sensors, we synthesized a new type of peptide probe for zinc ion. One strategy employed in the design of peptide probes for zinc is to link a fluorophore unit with a zinc binding motif. The two units are linked to each other in such a way that the binding of zinc ion to the binding motif causes considerable changes in the fluorescence. For easy synthesis, we designed a small peptide probe (5 mer) consisting of natural amino acids (Scheme 1). We chose a dansyl group as a fluorophore because the fluorescence emission of dansyl is sensitive to the microenvironment.[6] In addition, dansyl group has been used as a fluorophore in the previously synthesized peptide probes for monitoring Cu^{2+} and Pb^{2+}.[7] As dansyl fluorophore directly participated in the metal binding in these peptide probes, the metal binding event was effectively detected by fluorescence change. We designed zinc-binding motif of the peptide probes for the following reasons. As His, Glu, and Cys residues are frequently found as the ligands of zinc enzymes,[8] we designed peptide probes containing Cys, His, and Glu residues as a zinc binding amino acid. As a preorganization of the peptide ligand site may result in the increase of binding of metal ions[4j] we designed the peptides containing Pro-Gly amino acids to have a turn structure.[9] We developed **CP1** (dansyl-Cys-Pro-Gly-His-Glu) by synthesizing peptides containing His, Cys, and Glu residues in combination with Pro-Gly sequence. We also synthesized a control peptide (**P1**, dansyl-Gly-Gly-Gly-Gly-Gly).

Peptide probes were synthesized by the Fmoc-chemistry in solid phase peptide synthesis according to the literature procedure.[10] As **CP1** has good solubility, we investigated the fluorescence change in physiological buffer solution without cosolvent. We measured the fluorescence emission spectra of **CP1** in the presence of Zn^{2+}. Fluorescence spectra ranging from 350 to 650 nm were obtained by excitation with 330 nm. Upon Zn^{2+} addition, the emission intensity at 510 nm increased (Fig. 1a). The maximum emission wavelength was slightly shifted from 525 to 513 nm. This blue shift probably resulted from the dansyl fluorophore moving to a less polar environment upon metal binding.[6] In the titration curve, about 8 μM of Zn^{2+} was required for the saturation of **CP1**'s emission intensity. However, addition of Zn^{2+} to the control peptide **P1**, which is incapable



Scheme 1. Chemical structures of peptide probe and peptide–resin conjugate.

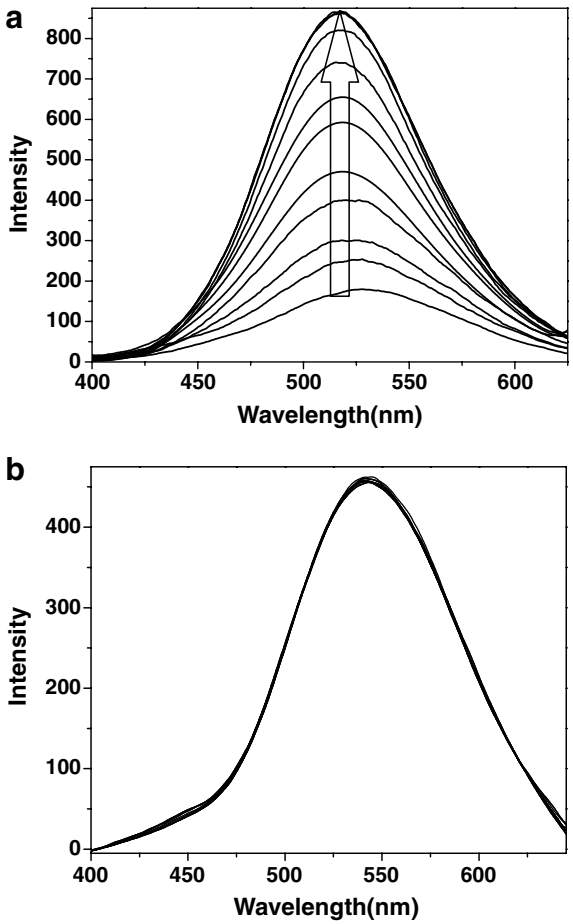


Figure 1. Fluorescence emission spectra of (a) **CP1** and (b) **P1** in the presence of Zn^{2+} (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μM). The emission spectra (λ_{ex} = 330 nm) of peptide probes (10 μM) were measured at 22 °C in 10 mM HEPES buffer (pH 7.4).

of binding metal, resulted in no fluorescence change (Fig. 1b).

To test the reversibility of **CP1**, 10 μM of EDTA was added to the peptide–Zn complex that exhibited a strong emission intensity. This addition to the peptide–Zn^{2+} complex instantly resulted in the return of the original, zinc-free spectrum, which demonstrates the readily reversibility of the signaling mechanism of the peptide (Fig. 2). Job plot analysis was carried out to determine the binding stoichiometry of the probe (data not shown). The 0.5 mole fraction at the maximum point indicated that **CP1** may form a 1:1 complex in micromolar range of peptide concentration. K_d value was calculated by using the ENZFITTER program based on the titration curve with Zn^{2+}.[11] The dissociation constant ($K_d = 1.4 \times 10^{-6}$ M) indicated that the probe can be a potential candidate to detect Zn^{2+} in the micromolar range.

We investigated the pH influence on the fluorescence intensity of the peptide in the absence and presence of Zn^{2+} (Fig. 3). At pH lower than 5.5, the peptide exhibited very weak fluorescence intensity, regardless of the presence or absence of Zn^{2+}. Previously synthesized

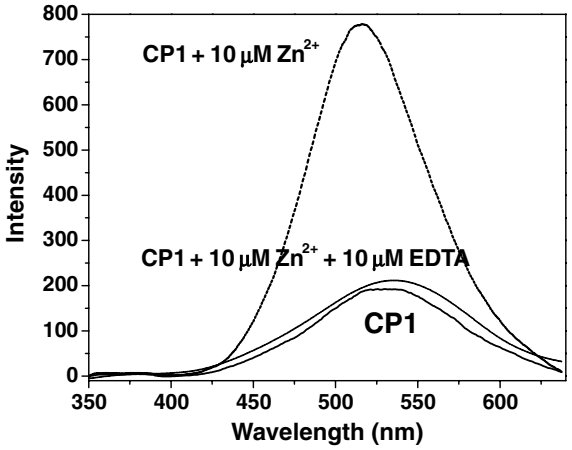


Figure 2. Fluorescence emission spectra of **CP1** in the presence of Zn^{2+} and EDTA. The emission spectra (λ_{ex} = 330 nm) of **CP1** (10 μM) were measured at 22 °C in 10 mM HEPES buffer (pH 7.4).

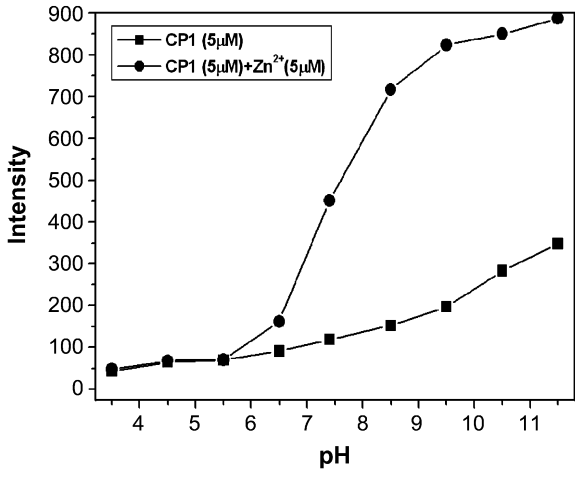


Figure 3. Fluorescence intensity of **CP1** at different pH values. The emission intensity at 520 nm was measured by excitation at a wavelength of 330 nm (slit size 2.5 and 4.5 nm).

probes containing dansyl moiety also showed a very weak emission intensity in acidic pH, which was explained by protonation of the dimethylamino group ($pK_a \sim 4$) of the dansyl fluorophore.[6a,12] At pH > 6.5, the intensity difference between **CP1** and **CP1**–Zn^{2+} complex increased with increasing pH, however the emission intensity of the peptide probe in the absence of Zn^{2+} was not considerably changed. Considering pK_a values of His and Cys residues, the negative charge of side groups of His and Cys residues must increase with increasing pH, which might enhance the interactions between the peptide and Zn^{2+}, resulting in the fluorescence increase of the probe in the presence of Zn^{2+} in this condition. At pH > 9.5, the intensity of free **CP1** slightly increased with increasing pH. This might be due to the deprotonation of the nitrogen atom of the sulfonamide group ($pK_a \sim 10$), which increased the electron density on the naphthyl ring. In addition, the increase of negative charge of the nitrogen atom might promote complexation of **CP1**–Zn^{2+} complex, resulting in the slight increase of intensity at pH > 9.5.

The fluorescence response of the peptide sensor in the presence of each metal ion (Ca^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+}, Cu^{2+}, Ag^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} as perchlorate anion and Na^{+}, Al^{3+}, K^{+}, as chloride anion) was investigated. Figure 4 shows the fluorescence emission change of the probe upon the addition of each metal ion at pH 7.4. The probe did not exhibit a fluorescence response with almost any of the metal ions except Cd^{2+} and Cu^{2+}. Specially, the zinc-dependent fluorescence was not affected by the presence of a 5 mM concentration of biologically important metal ions such as Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}. Generally, chemical or peptide probes are rarely able to differentiate between Zn^{2+} and Cd^{2+}.[4g,i] As the concentration of Cd^{2+} in biological systems is generally low compared to Zn^{2+} and the probe had a more potent binding affinity for Zn^{2+} ($K_d = 1.4 \times 10^{-6}$ M) than Cd^{2+} ($K_d = 2.3 \times 10^{-6}$ M), **CP1** can be applied for monitoring Zn^{2+} in biological system. Interestingly, the fluorescence intensity of **CP1** vanished in the presence of Cu^{2+}. Generally, other chemical and peptide probes containing dansyl moiety have strong binding affinities for Cu^{2+}, resulting in the decrease of emission intensity.[6,12a] Our control peptide, **P1**, also showed the considerable decrease of emission intensity in the presence of 2–10 μM concentration of Cu^{2+} (data not shown).

To test the utility of the peptides in further application, **CP1** was immobilized on the solid support and the binding affinities for various metal ions were investigated. For this, we synthesized a **CP1**–resin conjugate by synthesizing the peptide probe on PEG–PS–resin in consideration of the water swelling characteristics of PEG–PS–resin[13] (Scheme 1). The fluorescence emission of the con-

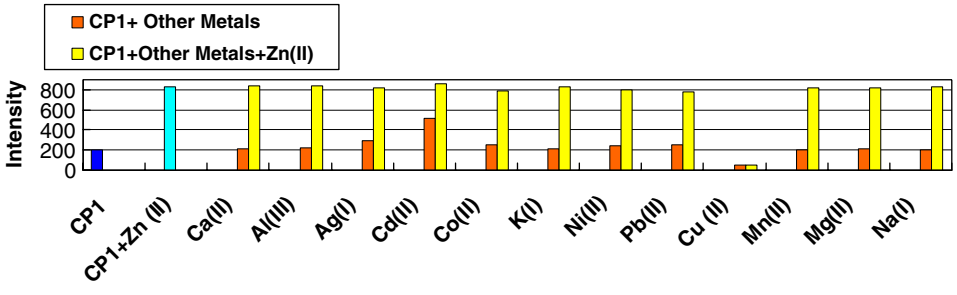


Figure 4. Fluorescence response of **CP1** (10 μM) in the presence of Zn^{2+} (10 μM) and/or various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions were evaluated at one equivalent to zinc ion except Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}, which were used at 5 mM.

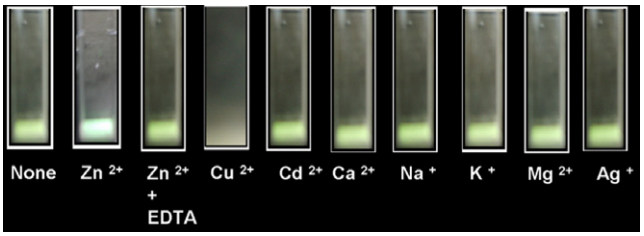


Figure 5. Fluorescence response of **CP1**–resin conjugate (300 μg/ml) in the presence of various metal ions at pH 8.4 (10 mM HEPES buffer). All metal ion concentration was 100 μM except for Na^+, K^+, Ca^{2+}, and Mg^{2+}, which were used at 5 mM. The concentration of EDTA was 100 μM. Fluorescence excitation was provided with a handheld UV lamp (ENF 260) set on long wavelength (365 nm). The image was acquired after 30 min of incubation.

jugate with various metal ions was measured in 10 mM HEPES buffer (pH 8.4). Figure 5 indicates that **CP1**–resin conjugate showed a considerable emission intensity increase only in the presence of Zn^{2+}, whereas the conjugate lost its original emission intensity in the presence of Cu^{2+}. The addition of EDTA to the resin–Zn complex that exhibited strong emission intensity resulted in the return of the original zinc-free intensity. This result confirmed that immobilized peptide similar to **CP1** displayed a great selectivity for Zn^{2+} and Cu^{2+} in the presence of several transition metal ions and this conjugate was easily regenerated through simple washing procedures with or without EDTA solution. In the present study, we successfully synthesized a novel peptide sensor for monitoring Zn^{2+}. As Zn^{2+} plays important role in many biological processes, various fluorescent peptide and chemical probes for monitoring Zn^{2+} have been developed.[4,14,15] Among them, commercially available 6-methoxy-8-*p*-toluenesulfonamido-quinoline (TSQ) is the most common and first applied for the measurement of cellular Zn^{2+}.[14] However, TSQ suffered limitations such as poor solubility in buffer system and tight binding affinity (subnanomolar affinity), while **CP1** has several advantages like good solubility in buffer system, micromolar affinity, and easy conjugation with solid support. However, **CP1** was too hydrophilic to penetrate into cells and it is useful for monitoring extracellular or environmental Zn^{2+}. In comparison to the previously reported fluorescence peptides,[4j] **CP1** showed similar pH sensitivity, selectivity, and binding affinity, which indicated that **CP1** was suitable for monitoring micromolar concentration of Zn^{2+}. **CP1** has some advantages over the previously reported peptide probes such as easy synthesis and tunable nature for controlling binding affinity because **CP1** consisted of natural amino acids.

In conclusion, we designed and synthesized a short fluorescence peptide probe consisting of natural amino acids with fluorophore and investigated its sensing ability for Zn^{2+}. The peptide probe that has a unique amino acid sequence, selectively and reversibly monitored Zn^{2+} in the presence of several other transition metal ions in aqueous solution. The peptide probe with micromolar affinity had a high selectivity for Zn^{2+} and formed a 1:1 complexation with Zn^{2+}. The peptide probe developed in this study can be a promising chemical sensor as diagnostic devices and for monitoring extracellular and environmental Zn^{2+} concentration.

Acknowledgments

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References and notes

1. (a) Falchuk, K. H. *Mol. Cell. Biochem.* **1998**, *188*, 41; (b) Zalewski, P. D.; Forbes, I. J.; Betts, W. H. *Biochem. J.* **1993**, *296*, 403; (c) Maret, W.; Jacob, C.; Vallee, B. L.; Fischer, E. H. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1936; (d) Cuajungco, M. P.; Lees, G. J. *Neurobiol. Dis.* **1997**, *4*, 137; (e) Choi, D. W.; Koh, J. Y. *Annu. Rev. Neurosci.* **1998**, *21*, 347.
2. Bush, A. I.; Pattingel, W. H.; Multhaup, G.; Paradis, M. D.; Vonsattel, J. P.; Gusella, J. F.; Beyreuther, K.; Masters, C. L.; Tanzi, R. E. *Science* **1994**, *265*, 1464.
3. Lippard, S. J.; Berg, J. M. *Principles of Bioinorganic Chemistry*; University Science Books: Mill Valley, CA, 1994, pp 10, 14, 78–183.
4. (a) Ajayaghosh, A.; Carol, P.; Sreejith, S. *J. Am. Chem. Soc.* **2004**, *127*, 14962, and reference therein; (b) Frederickson, C. J.; Bush, A. I. *Biometals* **2001**, *14*, 353; (c) Nolan, E. M.; Jaworski, J.; Okamoto, K.; Hayashi, Y.; Sheng, M.; Lippard, S. J. *J. Am. Chem. Soc.* **2005**, *127*, 16812; (d) Hanaoka, K.; Kikuchi, K.; Kojima, H.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 12470; (e) Park, M. S.; Swamy, K. M. K.; Lee, Y. J.; Lee, H. N.; Jang, Y. J.; Moon, Y. H.; Yoon, J. *Tetrahedron Lett.* **2006**, *47*, 8129; (f) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T. A.; Huxley, T. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515; (g) Kimura, E.; Koike, T. *Chem. Soc. Rev.* **1998**, *27*, 179; (h) Hirano, T.; Kicuchi, K.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *124*, 6555; (i) Walkup, G. K.; Imperiali, B. *J. Am. Chem. Soc.* **1996**, *118*, 3053; (j) Shults, M. D.; Pearce, D. A.; Imperiali, B. *J. Am. Chem. Soc.* **2003**, *125*, 10591; (k) Cheng, R. P.; Fischer, S. L.; Imperiali, B. *J. Am. Chem. Soc.* **1996**, *118*, 11349.
5. (a) Derossi, D.; Chassaing, G.; Prochiantz, A. *Trends Cell Biol.* **1998**, *8*, 84; (b) Deshayes, S.; Morris, M. C.; Divita, G.; Heitz, F. *Curr. Pharm. Des.* **2005**, *11*, 3629.
6. (a) Haughland, R. P. In *Handbook of Fluorescent Probes and Research Products*, 8th ed.; Molecular Probes, Inc.: Eugene, OR, 2001; (b) Lakowicz, J. R. *Principles of Fluorescence*; Plenum Press: New York, 1983.
7. (a) Zheng, Y.; Kerim, M.; Gattas, A.; Konka, V.; Roger, M. L. *Chem. Commun.* **2002**, *20*, 2350; (b) Deo, S.; Goldwin, A. G. *J. Am. Chem. Soc.* **2000**, *122*, 174; (c) Zheng, Y.; Cao, X.; Orbulescu, J.; Konka, V.; Andreopoulos, F. M.; Pham, S. M.; Leblanc, R. M. *Anal. Chem.* **2003**, *75*, 1706.
8. Lipscomb, W. N.; Strater, N. *Chem. Rev.* **1996**, *96*, 2375.
9. Cochran, A. G.; Tong, R. T.; Starovasnik, M. A.; Park, E. J.; McDowell, R. S.; Theaker, J. E.; Skelton, N. J. *J. Am. Chem. Soc.* **2001**, *123*, 625.
10. (a) Fields, G. B.; Nobel, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161; (b) To the resin bound peptide (65 mg, 0.05 mmol), dansyl chloride (40 mg, 0.15 mmol, 3 equiv)

in DMF (3 ml) and triethylamine (20 μl, 0.15 mmol, 3 equiv) were added. After cleavage of the product from resin, the peptides were purified from crude product by preparative HPLC with a C_{18} column. The success of synthesis and the purity (>95%) were confirmed by analytical HPLC with a C_{18} column (Manchester, UK) and a MALDI TOF mass spectrometer (Voyager-DE STR, Applied Biosystem).

11. Association constants were obtained using the computer program ENZFITTER, available from Elsevier-BIOSOFT, 68 Hills Road, Cambridge CB2 1LA, United Kingdom.
12. (a) Aoki, S.; Kawatani, H.; Goto, T.; Kimura, E.; Shiro, M. *J. Am. Chem. Soc.* **2001**, *123*, 1123; (b) O'Connor, N. A.; Sakata, S. T.; Zhu, H.; Shea, K. *Org. Lett.* **2006**, *8*, 1581.
13. Bayer, E. *Angew Chem. Int. Ed.* **1991**, *30*, 113.
14. Frederickson, C. J.; Kasarskis, E. J.; Ringo, D.; Frederickson, R. E. *J. Neurosci. Methods* **1987**, *20*, 91.
15. (a) Kikuchi1, K.; Komatsu, K.; Nagano, T. *Curr. Opin. Chem. Biol.* **2004**, *8*, 182; (b) Sarwar Nasir, M.; Fahrni, C. J.; Suhy, D. A.; Kolodsick, K. J.; Singer, C. P.; O'Halloran, T. V. *JBIC* **1999**, *4*, 775; (c) Fahrni, C. J.; O'Halloran, T. V. *J. Am. Chem. Soc.* **1999**, *121*, 11448.