A “Turn-On” Fluorescent Chemosensor Based on Peptidase for Detecting Copper(II)

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A new fluorescent chemosensor for Cu(II) ions was designed and synthesized on the basis of the sequence-specific cleavage of the peptide bond by the peptidase (metal or metal complexes). In the chemosensor system, the substrate was labeled with a FAM fluorophore (6-carboxyfluorescein) at the N-terminal and with a Dabcyl quencher 4-(4'-dimethylaminophenylazo)benzoic acid at the ϵ -N of C-terminal Lys. In the presence of Cu(II), the substrate strand is cleaved, and the release of the cleaved fragment results in a significant fluorescence increase. The design was aided by the FRET study that showed a “turn-on” response for Cu(II) in an aqueous medium. Under optimum conditions, the novel chemosensor described here had a linear response range for Cu(II) from 1.0×10^{-8} to 1.0×10^{-6} mol dm⁻³ with a detection limit of 1.0×10^{-8} mol dm⁻³.

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Introduction

Fluorescent chemosensors for the detection and measurement of transition-metal ions are widely investigated because of their simplicity and high sensitivity of response.¹ The determination of heavy metals in the aquatic environment is of tremendous interest due to their hazardous effects on the ecosystem and on human health, depending on the dose and the toxicity. Among the metal ions, copper is a widely used metal that can leak into the environment through various routes. Low concentrations of copper are an essential nutrient. However, exposure to high levels of copper even for a short period of time can cause gastrointestinal disturbance, while long term exposure causes liver or kidney damage.² The U.S. Environmental Protection Agency (EPA) has set the limit of copper in drinking water to be 1.3 ppm (~20 μ M). Therefore, the trace copper content in water and food must be controlled on a daily basis. Copper could be determined by inductively coupled plasma mass spectrometry (ICP-MS), or electrothermal atomic absorption spectrometry (ETAAS), which usually have a sufficiently low detection limit. Flame atomic absorption spectrometry (FAAS) is available in most laboratories, but it requires the use of a preconcentration step in order to reach an appropriate level of sensitivity.³ With regard to sensitivity and accuracy, all of these methods are efficient tools for heavy metal ion determination; however, they are expensive, and/or require sophisticated manipulation. Therefore, the development of a simple and inexpensive chemosensor for the detection of transition metal ions remains desirable for the real-time monitoring of environmental, biological, and industrial samples.⁴⁻⁷ Moreover, research about

fluorescent chemosensors has become a rapidly expanding area of analytical chemistry, because they offer the advantages of simple preparation, reasonable selectivity and sensitivity.

In the past decade, considerable attention has been focused on the design of fluorescent chemosensors for Cu(II) ion due to the highly sensitive, quick, and nondestructive advantages of the fluorescent method.⁸ A widely used strategy is to link the metal recognition portion closely with a signal generation moiety such as a fluorophore. While quite successful in designing sensors for diamagnetic metal ions such as Pb(II), Hg(II), Zn(II), and Cu(I), this method has been applied to paramagnetic metal ions such as Cu(II) with only limited success, due to their intrinsic fluorescence quenching properties. Most Cu(II) chemosensors showed decreased emission upon Cu(II) binding, which was undesirable for analytical purposes. First, the room for signal change was at most 1-fold. Second, such “turn-off” chemosensors may give false positive results due to quenchers in real samples.⁹ Furthermore, only a few examples can display “turn-on” or ratiometric fluorescent changes in emission spectra, which are desirable for analytical purposes due to the enhancement of fluorescence or changes in the ratio of the intensities of the emission at two wavelengths.

The hydrolytic chemistry of peptide bonds has been used with more success in terms of sequence specificity. Many metal ions and metal complexes are found to promote or catalyze the hydrolysis of peptide bonds in short peptides.¹⁰⁻¹⁵ Bal's group identified the specific Ni(II)-related hydrolysis of peptide bonds preceding Ser/Thr-Xaa-His sequences.¹⁶⁻¹⁸ In previous studies, the specific Cu(II)-related hydrolysis was demonstrated for peptide bonds preceding Ser-His and Thr-His sequences at elevated temperatures and pH values.¹⁹⁻²² These studies indicated that such hydrolysis is capable of transducing signals through both molecular recognition of metal ions and metal-independent hydrolytic cleavage activity, resulting in a potentially wide

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detection range and high sensitivity. Recently, we have reported a new application for metal-dependent peptidase as a unique class of chemosensors for Ni(II) ions.²³ Keeping the above strategies in mind, we designed a Cu(II) chemosensor based on peptidase, which displayed turn-on fluorescence with high sensitivity for Cu(II) in an aqueous medium, with a quantifiable detection range from 10 nM to 5 μ M.

Experimental

Materials and chemicals

Cu(II) chemosensor peptidase (CCP) was synthesized by Gelsion Inc. (Shanghai, China), purified by HPLC with over 98% purity, and characterized by ESI-MS. All other chemicals were of analytical grade and were used without further purification. All solutions were prepared with Milli-Q water (Milli-Q, Millipore, USA, 18.2 M Ω cm⁻¹ resistivity).

All spectroscopic measurements were performed in a buffer of 1000 mm³ (fluorometer assays) volume containing 1 μ M CCP, 50 μ M H₂O₂ and 20 mM phosphate buffer, pH 9.0. The stock solution of CCP (1 mM) was prepared in Milli-Q water, stored in the dark at 4°C, and diluted to the required concentration (1.43 μ M) with Milli-Q water.

Fluorometer assays

In a typical experiment, 700 mm³ of CCP (1.43 μ M) were added to a buffer of 1000 mm³ volume containing 100 mm³ 200 mM phosphate buffer, 100 mm³ 500 μ M H₂O₂ and 100 mm³ metal ion stock solutions (10 times stock solution). The final CCP concentration was 1 μ M, the final phosphate buffer concentration was 20 mM and the final H₂O₂ concentration was 50 μ M. The above prepared mixtures were vortexed to mix and then incubated in a water bath of 62°C for 60 min and allowed to cool to room temperature in ice cold water for 5 min. The above prepared cuvette was vortexed to mix all the reagents and placed into a fluorometer (Hitachi F-4500 fluorescence spectrophotometer, Japan). The cuvette was allowed to incubate in the cuvette holder of the fluorometer for 2 min before taking any measurement. An excitation wavelength of 490 nm (slit width, 2.5 nm) was used, and data were collected over 500–620 nm (slit width, 10 nm).

Results and Discussion

The ^NDKTHC sequence was cleaved by Cu(II) when present in a peptide (as suggested by Smith *et al.*).^{19,20} The sequence was cleaved between the Lys and Thr residues. The degree of cleavage increased with increasing temperature, length of time and pH of incubation, and also with increasing amounts of available Cu(II) ions.^{21,22} Based on the original Cu(II)-dependent peptide sequences reported by Smith *et al.*,^{19,20} we rationally designed a Cu(II) chemosensor peptidase (CCP) as shown in Fig. 1. The chemosensor contained a basic specific Cu(II)-dependent peptide bond hydrolysis sequence -(Ser-Asp-Lys-Ser-His-Thr-Lys). The substrate (in black) was labeled with a FAM fluorophore (6-carboxyfluorescein) at the N-terminal and a Dabcyl quencher 4-(4'-dimethylaminophenylazo)benzoic acid at the ϵ -N of C-terminal Lys. Initially, the FAM emission was quenched by the nearby quencher. In the presence of Cu(II), the substrate was irreversibly cleaved at the cleavage site between the Lys and Ser of -(Ser-Asp-Lys-Ser-His-Thr-Lys), which is confirmed by chromatography data (Fig. S1, Supporting Information). Upon cleavage, the fluorophore was released and

Fig. 1 The sequence and structure sketch of the Cu(II) chemosensor peptidase (CCP). F and Q denote fluorophore and quencher, respectively.

became located far away from the quencher, leading to its increased fluorescence. Fluorescence spectra were obtained in 20 mM phosphate buffer with pH 9.0 and 50 μ M H₂O₂ by exciting into the FAM fluorophore absorption at 490 nm and scanning the emission from 500 to 620 nm. Upon addition of Cu(II), the fluorescence intensity of FAM increases by *ca.* 16-fold FAM (Fig. 2a) with the same absorption ($\lambda_{\text{abs}} = 490$ nm) and emission maxima ($\lambda_{\text{em}} = 517$ nm) as the apo chemosensor, which support a previous hypothesis that the fluorophore FAM was released and became located far away from the quencher. According to our supporting experiment, 20 mM phosphate buffer (Fig. S2) and pH 9.0 (Fig. S3) were chosen for further experiments to obtain fast hydrolysis rates, high fluorescence intensity, and good solubility under alkaline conditions. The chemosensor system also contained 50 μ M H₂O₂, because it can significantly enhance the reaction rate (Fig. S4).

To test sensitivity, we monitored fluorescence intensity increases at 517 nm in the presence of varying concentrations of Cu(II). The fluorescence emission properties of FAM are sensitive to the presence of nanomolar concentrations of Cu(II). As shown in Fig. 2a, depending on the Cu(II) concentration range, there are different response sensitivities. The fitting curve of the inset of Fig. 2b can serve as the calibration curve for the detection of Cu(II) concentration. The linear range of Cu(II) was 1.0×10^{-8} to 1.0×10^{-6} mol dm⁻³. The correlation coefficient (R^2) was 0.9973, showing a good linearity of calibration curve. According to the definitions of the International Union of Pure and Applied Chemistry, the detection limit of the chemosensor was calculated based on three times of the standard deviation of 11 runs of the blank solution. A detection limit of 10 nM was determined, which represents one of the most sensitive turn-on Cu(II) chemosensors that we know of. The chemosensor also has a dynamic range of up to 5 μ M, which is useful for detecting Cu(II) in drinking water because the U.S. EPA has defined a maximum contamination level of 20 μ M.

We have further evaluated the selectivity of our system for metal ions (Fig. 3). The tested metal ions representative of (Ba(II), Ni(II), Mg(II), Co(II), Zn(II), Mn(II), Fe(II), and Ca(II)) were added to the solution of CCP, respectively; each mixture was tested under the same conditions as in the case of Cu(II). The spectra showed negligible changes when the metal ions were each added to the solution of CCP. The competition experiments of Cu(II) and other metal ions were also performed.

Fig. 2 Fluorescence response of 1 μM CCP to Cu(II). (a) Superimposed are emission fluorescence spectra of FAM at various Cu(II) concentrations. Spectra were acquired in 20 mM phosphate buffer, pH 9.0 and 50 μM H_2O_2 , with excitation at 490 nm. (b) The fluorescence intensity plotted against Cu(II) concentration. Inset: intensities at the low Cu(II) region. Error bars represent standard deviations from three repeated experiments.

Fig. 3 Fluorescence responses of CCP (1 μM) to 1 μM of Cu(II) or 10 μM of other metal ions and to the mixture of 10 μM of other divalent metal ions with 1 μM of Cu(II). Spectra were acquired in 20 mM phosphate buffer (pH 9.0) and 50 μM H_2O_2 . Bars represent the final (F) over the initial (F_0) integrated emission. White bars represent the addition of the competing metal ion to a 1 μM solution of CCP. Black bars represent the subsequent addition of 1 μM Cu(II) ion to the solution. Excitation was provided at 490 nm, with emission integrated over 500 – 620 nm. Standard deviation represents measurements on three distinct surfaces.

As shown in Fig. 3, when the mixed solution of Cu(II) ion (1 μM) with other metal ions (10 μM) were added to the solution of CCP (1 μM), respectively, only Mn(II) and Fe(II) ions had a slight disturbance when present in much excess. The results indicate that the binding of Cu(II) ion to CCP is much stronger than that of other metal ions, though the concentrations of the other metal ions are ten-fold to Cu(II) ion. Therefore, the interference from those metal ions could be neglected and CCP can be considered as a high selective fluorescence chemosensor for Cu(II) ion with low detection limit. All these selective results indicate that our proposed chemosensor could meet the selective requirements for biomedical and environmental applications. Given its desirable sensitivity and selectivity, our molecular engineering design may prove useful in the future development of other peptidase-based chemosensors for toxicological and environmental monitoring.

Conclusions

In summary, we have described a fluorescence turn-on chemosensor for Cu(II) based on metal-dependent hydrolysis of peptide. It combines the high selectivity of a peptide designed for the Cu(II) ion with the high sensitivity afforded by the fluorophore attached to the peptide. The fluorescent chemosensor showed strong fluorescence enhancement in the presence of a paramagnetic metal ion: Cu(II). Under optimum conditions, the novel chemosensor described here had a linear response range for Cu(II) from 1.0×10^{-8} to 1.0×10^{-6} mol dm^{-3} with a detection limit of 1.0×10^{-8} mol dm^{-3} . Since the fluorescence domain is decoupled from the metal-recognition catalysis domain, the sensitivity and selectivity of this system can be further improved separately. As most transition-metal ions possess intrinsic fluorescence quenching properties, it is very challenging to develop their chemosensors with fluorescence enhancement.²⁴ However, alternatively, the design approach for this chemosensor should lead to the development of fluorescence turn-on chemosensors for other transition metal ions by employing a particular metal-facilitated reaction.

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Supporting Information

Figures S1 – S5 are in the material. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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