Biol Trace Elem Res (2010) 134:352–363
DOI 10.1007/s12011-009-8476-9

Fluorescent Protein-Based Optical Biosensor for Copper Ion Quantitation

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Received: 10 May 2009 / Accepted: 17 July 2009 /
Published online: 1 August 2009

Abstract In the present study, spectroscopic determinations of copper ions using chimeric metal-binding green fluorescent protein (His6GFP) as an active indicator have been explored. Supplementation of copper ions to the GFP solution led to a remarkable decrease of fluorescent intensity corresponding to metal concentrations. For circumstances, rapid declining of fluorescence up to 60% was detected in the presence of 500 μM copper. This is in contrast to those observed in the case of zinc and calcium ions, in which approximately 10–20% of fluorescence was affected. Recovery of its original fluorescence up to 80% was mediated by the addition of ethylenediamine tetraacetic acid. More importantly, in the presence of metal ions, the emission wavelength maximum remains unchanged while reduction of the optical density of the absorption spectrum has been observed. This indicates that the chromophore's ground state was possibly affected by the static quenching process. Results from circular dichroism measurements revealed that the overall patterns of circular dichroism spectra after exposure to copper ions were not significantly different from that of the control, where the majority of sharp positive band around 195–196 nm in combination with a broad negative deflection around 215–216 nm was obtained. Taken together, it can be presumed that copper ions exerted their static quenching on the fluorescence rather than structural or conformational alteration. However, notification has to be made that some peptide rearrangements may also occur in the presence of metal ions. Further studies were conducted to investigate the feasibility of using the His6GFP as a sensing unit for copper ions. The His6GFP was encapsulated in Sol-gel and immobilized onto the optical fiber connected with a fluorescence detecting device. The Sol-gel was doped into the metal solution where the quenching of fluorescence could be monitored in real time. The sensing unit provided a high sensitivity of detection in the range of 0.5 μM to

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50 mM with high selectivity for copper ions. All these findings open up a high potential to apply the fluorescent protein-based bioanalytical tool for copper determination in the future.

Keywords Copper ions · Green fluorescent protein · Sol-gel · Optical sensor

Introduction

Copper is an essential trace element possessing several important roles in biological processes, e.g., electron transfer in cellular respiration, cofactor of oxidative scavenging enzymes, and as redox-regulating components [1]. Dietary intake of copper at appropriate amount is required (a) to maintain the normal formation of brain and nervous systems, (b) to sustain the elasticity of blood vessels, (c) to mediate formation of collagen and elastin, and (d) to regulate hemoglobin levels [2]. Imbalance of copper homeostasis results in many pathological consequences such as Menkes and Wilson's diseases, tumor development and progression, and brain disorders [3]. Determination of copper levels in both biological and environmental samples normally exploits atomic absorption spectroscopy and inductively coupled plasma mass spectroscopy, which are complicated and sample destructive. Therefore, several attempts have been geared towards the utilization of ion-selective electrode, electrochemistry, and synthetic metal-binding molecules as tools for copper detection [4–6]. Recently, the fluorescent proteins from various organisms become potential candidates for sensor development. These include the green fluorescent protein (GFP) and its variants from the Pacific Northwest jellyfish namely *Aequorea victoria* [7–11], the red fluorescent protein (DsRed) from the tropical coral namely *Discosoma* sp. [12–14], and the far-red fluorescent (HcRed) protein from the reef coral namely *Heteractis crispa* [15, 16].

GFP is an autofluorescent protein where its greenish fluorescence is emitted at 509 nm upon excitation at 395 nm or exposed to blue light (for recent review, please see [17]). Fluorescent emission is generated by cyclization of tripeptide (Ser^{65}, Tyr^{66}, Gly^{67}) followed by dehydration and oxidation of the chromophore [18]. Neither substrate nor cofactor is required for the fluorescent emission. The GFP has extensively been applied as a reporter for gene expression and protein localization, as pH indicators for in vivo uses, as tagger for immunological diagnosis, and as tools for biosensor applications [19–22]. To serve as a biosensor for metal determination, a chimeric GFP harboring hexahistidine has been engineered and investigated the changes of its fluorescence in the presence of divalent metal ions since 2000 [7]. By the same year, Richmond and his colleagues have introduced metal-binding sites onto the surface of GFP and found that metals in close proximity to chromophores are known to quench fluorescence in a distance-dependent fashion [8]. In 2001, whole *Escherichia coli* cells expressing a chimeric cadmium-binding peptide fused to GFP have been applied for determination of cadmium ions [9]. Emphasis can be taken that cadmium and zinc ions enhance fluorescent emission of the engineered cells in a dose-dependent manner whereas a rapid declining of fluorescence is found in the presence of copper ions. One year later, a green fluorescent protein mutant (BFPms1) has been used as Zn biosensor [10]. Binding to zinc ions (K_d~50 μM) of the BFPms1 results in a fluorescent enhancement while copper ions (K_d~24 μM) exert their quenching effects on the fluorescent emission. During 2004–2005, several biophysical studies have been conducted to investigate the interaction between chimeric metal-binding GFPs and artificial lipid membranes for further development of membrane-based metal sensor [23–26]. Additionally, engineered *E. coli* co-expressing surface exposed zinc-binding motif and GFP has been applied for real-time monitoring of intracellular mobility of zinc ions [27]. In 2007, a novel

solvent-exposed analyte channel, generated by site-directed mutagenesis of F165G, on the surface of GFP has been constructed [11]. This mutant exhibits the highest sensitivity towards copper ions at physiological pH with the dissociation constant of ~4.9 μM. Recently, three GFP mutants (N146H, F165H, and L201H) have been engineered for metal-binding studies [28]. However, it is worth to mention that none of the GFP variants has been incorporated with the detecting unit and applied for biosensor purposes. In addition, alterations of fluorescent responses of GFP as a consequence of metal ions, in particular copper and zinc, remain not fully understood.

Therefore, in the present study, spectroscopic studies (e.g., fluorescence measurements and circular dichroism measurements) of chimeric green fluorescent protein harboring hexahistidine, designated as His6GFP, in the absence and presence of metal ions have been carried out. Next, encapsulation of the His6GFP in Sol-gel has been performed. The encapsulated material has subsequently been immobilized onto the optical fiber and applied for instantaneous determination of copper ions in the assay solution.

Materials and Methods

Materials

Chimeric green fluorescent protein harboring hexahistidine, designated as His6GFP [9, 23, 27], was expressed in *E. coli* strain TG1 and purified to homogeneity (>95% purity) using single-step immobilized metal (Zn^{2+}) affinity chromatography as previously described. All reagents and chemicals used in this study are of analytical grade and commercially available.

Fluorescence Spectroscopy Measurements

Fluorescence measurements were carried out using a Perkin Elmer LB-50 spectrofluorometer at 298 K. Excitation and emission wavelengths were assayed at 395 and 509 nm, respectively. Samples containing 0.5 μM His6GFP in 50 mM Na_2HPO_4 and 300 mM NaCl, pH7.4 were mixed with $CaCl_2$, $ZnSO_4$, or $CuSO_4$ dissolved in the same buffer to yield the final concentrations in the range of 0.5–500 μM before fluorescence scanning or measurements using a cuvette with 1-cm path length. In some circumstances, the addition of ethylenediamine tetraacetic acid (EDTA) to the sample solution (at final concentration of 1 mM) was performed for comparison.

Circular Dichroism Spectroscopy Measurements

Circular dichroism measurements were acquired using a Jasco J-600 spectrometer at 298 K. Samples (300 μl) were analyzed in quartz cells with path length of 0.1 cm. Far-UV circular dichroism (CD) spectra were recorded between 190 and 260 nm with 1 nm resolution, scan rate of 100 nm/min, sensitivity of 20 mdeg, bandwidth of 1 nm, and response time of 0.5 s. All the spectra were corrected by subtraction of the background buffer solution. Typically, four spectra were accumulated and subsequently averaged. The ellipticity results were expressed as mean residue ellipticity, $[\theta]$, in degrees square centimeter per decimole as calculated by the following equation:

$$[\theta] = (\theta)/(10lC)$$

where (θ) is the ellipticity in degrees (deg), l is the path length in centimeters (0.1 cm), and C is the molar concentration per residue, that is, the product of the molar protein concentration (5×10^{-6}M) and the number of residues (261 amino acids).

Samples containing 5 μM His6GFP in 5 mM Na_2HPO_4 and 30 mM NaCl, pH7.4 were mixed with $CaCl_2$, $ZnSO_4$, or $CuSO_4$ dissolved in the same buffer to yield the final concentrations of 5–500 μM. The solution was allowed to equilibrate for 10 min before CD measurements. In some circumstances, the addition of EDTA to the sample solution (at a final concentration of 1 mM) was performed for comparison.

Encapsulation of His6GFP in Sol-gel and Determination of Copper Ions via Protein-Based Optical Sensor

To encapsulate the His6GFP in Sol-gel, three components including tetramethoxysilane (TMOS), distilled water, and 0.1 N HCl were mixed together at the molar ratio of 1:5:0.01. The mixture was then vortexed until a clear solution was obtained. The solution was stored at 4°C for 48 h and served as the pre-polymerized Sol-gel. For the polymerization process, 50 μl of pre-polymerized Sol-gel was mixed with equal volume of 0.05 N NaOH. Then, 100 μl of the mixture was mixed with 100 μl protein solution (2 μM). The solution was vortexed and then 250 μl was poured onto a container. The solution was allowed to solidify at room temperature. After 5 min, the Sol-gel was subsequently equilibrated and kept in 10 ml 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) buffer (5 mM HEPES, 0.85% NaCl, pH7.1) at 4°C overnight.

To further set up a sensor unit for metal ions, the Sol-gel was put into a round Teflon trough connected with fiber optic. This optical unit was dipped into a beaker containing various concentrations of copper ions (0.5 μM to 50 mM) dissolved in HEPES buffer. Changes of fluorescent emission were real-time monitored at the interval time of 10 min for 1 h. A calibration curve for copper ions was then generated. The effects of various kinds of divalent cations (Mg^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, and Fe^{2+}) on the fluorescent responses were also compared. To reuse the sensor unit, the Sol-gel was equilibrated with 50 mM EDTA and washed several times with HEPES buffer.

Results and Discussion

Fluorescent Characteristics of His6GFP in the Presence of Copper Ions and Other Divalent Cations

Figure 1 illustrates the fluorescence excitation and emission spectra of purified His6GFP. An excitation wavelength maximum of 395–397 nm and an emission wavelength maximum of 509 nm corresponding to previous reports [11, 18] were observed. The effect of various concentrations (0.5 μM to 500 μM) of divalent cations, e.g., Ca^{2+}, Zn^{2+}, and Cu^{2+} on the fluorescent emission spectra of the His6GFP was investigated. As represented in Fig. 2, it has been suggested that copper ions exerted a strong suppressing effect on the fluorescent intensity corresponding to metal concentrations (Fig. 2c). The remaining fluorescence of approximately 40% was found in the presence of 500 μM $CuSO_4$. Meanwhile, up to 80% and 90% fluorescent intensity remained upon the addition of 500 μM $ZnSO_4$ and $CaCl_2$, respectively (Fig. 2b, a). Recovery of approximately 80% of its original fluorescence was demonstrated once chelation of copper ions by EDTA (1 mM) was carried out (Fig. 2d). More importantly, it is worth noting that the emission wavelength maximum remains

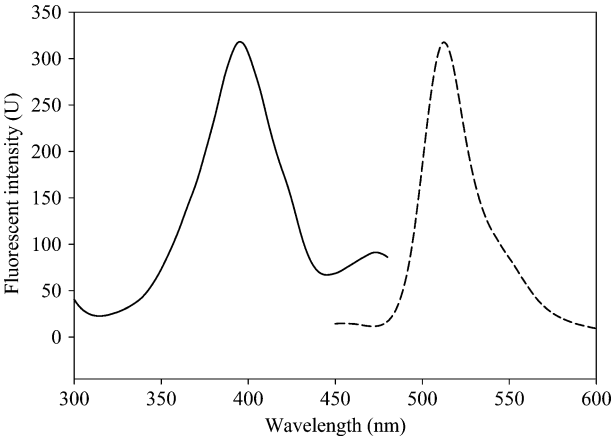

Fig. 1 Excitation (*solid line*) and emission (*dash line*) spectra of chimeric His6GFP

unchanged even in the presence of metal ions, which is consistent with previous reports [7, 8, 11, 29]. In addition, the absorption spectra in the presence and absence of copper were indistinguishable except for the diminution in the optical density upon supplementation with copper (data not shown). Therefore, our findings lend support to the notion that alteration of fluorescent emission was possibly mediated by metal-catalyzed fluorescence quenching. Typically, quenching of fluorescence can be caused by a variety of molecular interactions, e.g., ground-state complex formation, excited-state reaction, molecular

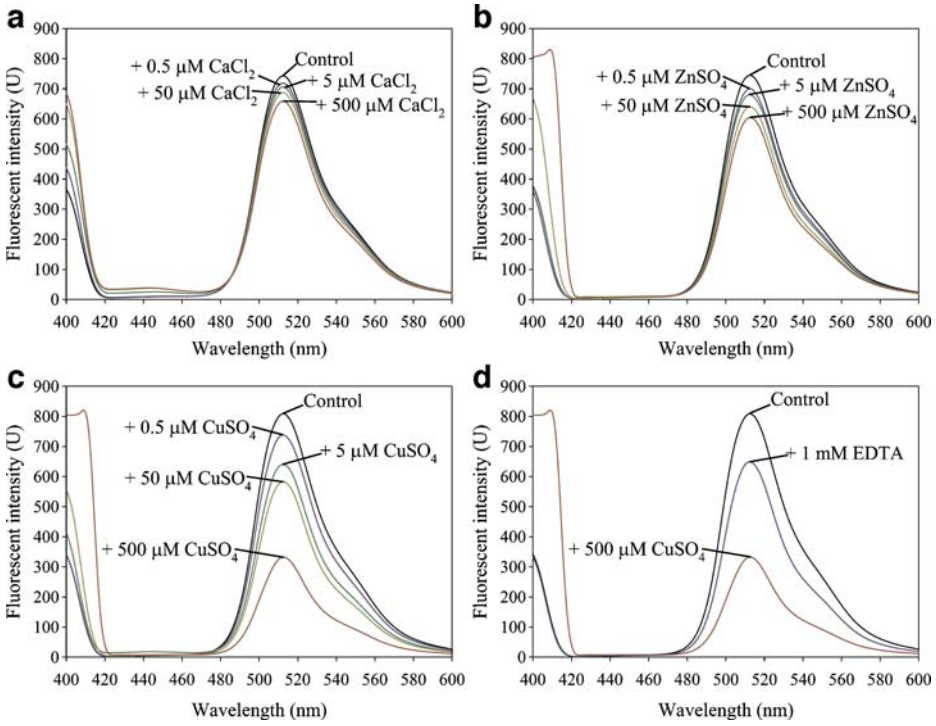

Fig. 2 Effects of various concentrations of calcium ions (**a**), zinc ions (**b**), or copper ions (**c**) on fluorescence spectra of His6GFP. Addition of EDTA to the protein solution after exposure to copper ions (**d**)

rearrangements, energy transfer, and collisional (dynamic) quenching. It is well established that various substances (molecular oxygen, iodide ion, acrylamide, peroxides, sulfur dioxide, etc.) can act as quencher of fluorescence. In our case, both the optical density of absorption peak and fluorescent emission intensity were influenced, indicating that the ground state of GFP's chromophore was affected by metal ions via static (contact) quenching process. This is unlike the collisional quenching in which alteration of the excited state of the chromophore has been ensued. Molecular oxygen is considered to be the best example for this type of quenching.

Dose–response curves were further generated for divalent cations by plotting the percentage of fluorescence quenching against concentrations of metal or calcium ions (Fig. 3). Copper ions (in the range of 0.5 μM to 5 mM) caused a remarkable decrease of fluorescent intensity. Exposure to a low dose (0.5 μM to 500 μM) of zinc and calcium ions resulted in the remaining fluorescence of more than 80%. In contrast, a steep peak of fluorescence loss was found at higher concentrations. Altogether, the sensitivity against divalent cations of the His6GFP was in the order of $Cu^{2+} >>> Zn^{2+} \gg Ca^{2+}$. To further analyze the selectivity towards copper ions, the mixed solution method was then applied in which the His6GFP was incubated with a mixed solution of 5 mM $CuSO_4$ and 5 mM $CaCl_2$ or $MgCl_2$ prior to fluorescent measurements. As illustrated in Fig. 4, our results revealed that no significant interference from calcium and magnesium ions was observed on the fluorescent quenching of His6GFP by copper ions. Our findings confirm that the quenching observed is mainly attributable to the static quenching since the quenching effect is very definite to copper binding (K_d~17 μM). Plausible explanations can be drawn that the presence of intramolecular histidine residues and the polyhistidine-tag at the N-terminus may facilitate the accumulation of copper ions in close proximity to the chromophore, where donation of an electron from the chromophore to copper ions and formation of a non-fluorescent ground state complex can occur. Other supportive evidences on the static quenching of HcRed fluorescence as a consequence of copper–protein complex have also been documented [15, 16].

Circular Dichroic Spectra of His6GFP in the Presence of Copper Ions and Other Divalent Cations

To identify the mechanism of quenching by copper ions, determination of CD spectra of the His6GFP upon metal addition was performed. Figure 5 shows the baseline CD spectrum (in

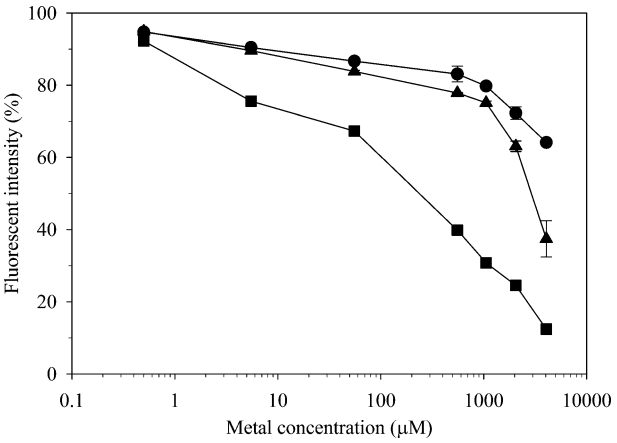

Fig. 3 Dose–response curves of His6GFP against calcium ions (*filled circle*), zinc ions (*filled triangle*), or copper ions (*filled square*). Data represent as mean ± SD (n=3)

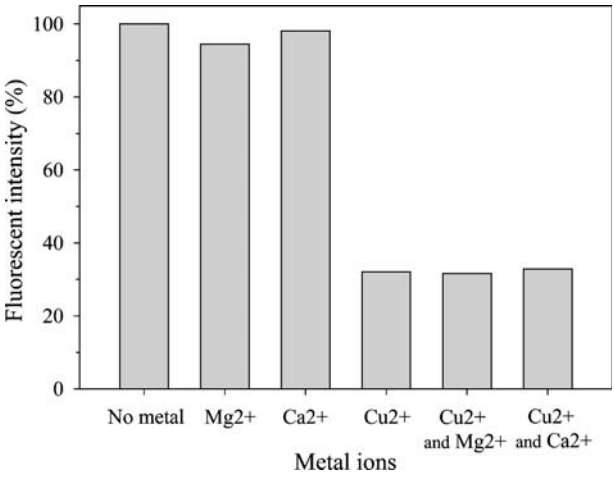

Fig. 4 Effect of divalent cations on fluorescent responses of chimeric His6GFP to copper ions

the far-UV range) of His6GFP in the absence of metal ions. The spectral profile of His6GFP was similar in shape to those reported for the wild-type GFP and its mutants [30, 31]. Generally, the α-helix structure is represented as a positive band at 192 nm and negative bands at 208 and 222 nm, while the β-sheet structure is found as positive and negative bands at 195 and 216 nm. Since the GFP consisted of 11 β-pleated sheets formed as the β-can structure [18]; therefore, the majority of sharp positive bands around 195–196 nm in combination with a broad negative deflection around 215–216 nm was observed (Fig. 5) [30]. Upon exposure to various concentrations of copper ions, it seemed that the overall patterns of CD spectra were not significantly different from that of the control; however, it should be mentioned that alterations on the spectra corresponding to metal concentrations were potentiated (Fig. 6c). The effect of zinc and calcium ions on the CD spectra was also found to be concentration-dependent but in lesser extents (Fig. 6b, a). The addition of EDTA brought about a recovery of negative band around 215–216 nm, but the majority of normal dichroic profile could be incompletely retrieved (Fig. 6d). Taken together with the aforementioned observation on fluorescent characteristics, it can be suggested that reduction of fluorescent emission as a consequence of copper ions is mainly

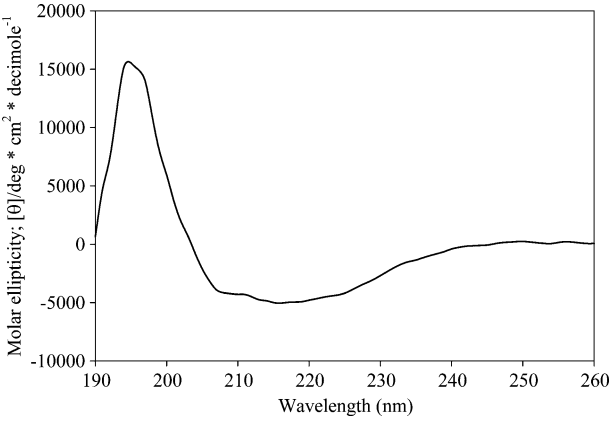

Fig. 5 Circular dichroic profile of His6GFP in 5 mM Na_2HPO_4 and 30 mM NaCl, pH7.4

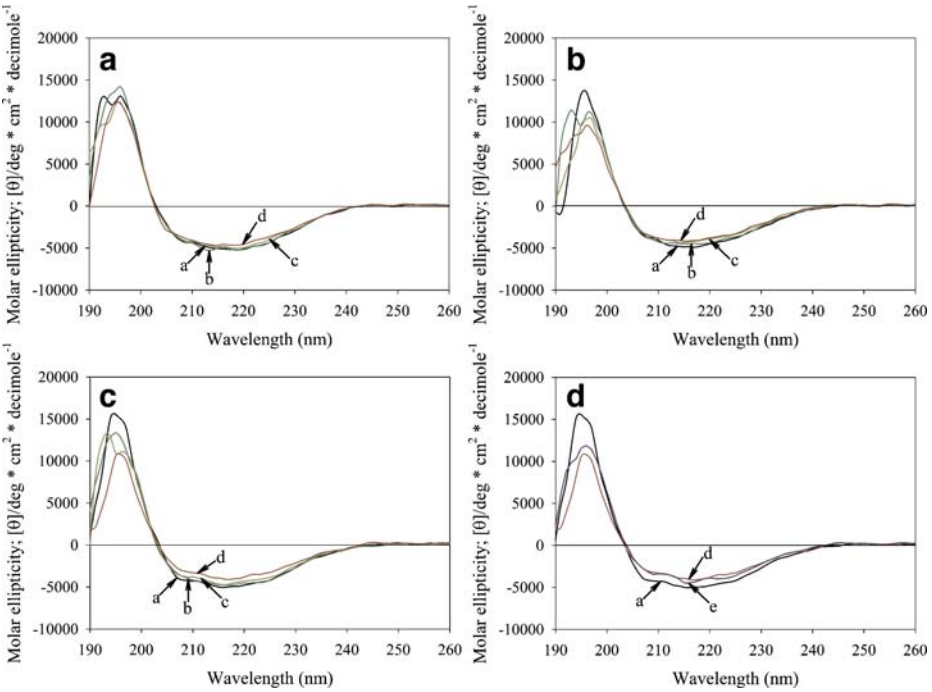

Fig. 6 Circular dichroic profiles of His6GFP in the presence of calcium ions (**a**), zinc ions (**b**), or copper ions (**c**). Addition of EDTA to the protein solution after exposure to copper ions (**d**). *a* The CD spectrum of His6GFP in the absence of divalent cations; *b–d* the CD spectra of His6GFP in the presence of 5, 50, and 500 μM of divalent cations, respectively; **e** the CD spectrum of His6GFP in the presence of 1 mM EDTA

caused by static quenching rather than structural or conformational changes. Conclusion from this finding gains insight into the changes of fluorescence of GFP by divalent cations observed in earlier studies [7, 8, 27]. However, it cannot be ruled out that in the presence of copper ions, some peptide rearrangements may also have taken place (Fig. 6c).

Development of Protein-Based Optical Sensor for Copper Determination

As schematically depicted in Fig. 7, development of protein-based metal sensor was carried out by encapsulating the His6GFP in Sol-gel and immobilizing on optical fibers. Quenching of fluorescent emission can be instantaneously monitored by excitation at 395 nm. It has been revealed that the percentage of TMOS influenced the levels of fluorescent quenching. As shown in Fig. 8, un-optimized condition would bring about a linear response in narrow range. Using the ratio of TMOS as stated in the "Materials and Methods" section, a broader range of dose–response curve against copper ions (from 0.5 μM to 50 mM) was attained (Fig. 9). Responses of the encapsulated His6GFP against other metal ions were further examined (Fig. 10). It can be emphasized that quenching of His6GFP fluorescence was more pronounced in the presence of Cu^{2+} and Fe^{2+}. Decrease of approximately 15–20% fluorescent intensity was observed in the case of Mn^{2+}, Cd^{2+}, and Zn^{2+}. As control, calcium and magnesium ions (commonly found in drinking water) exerted a minute effect on fluorescence. Such excellent selectivity to copper ions and the high sensitivity comparable to those of the fluorescent compounds (e.g., Lucifer Yellow [32],

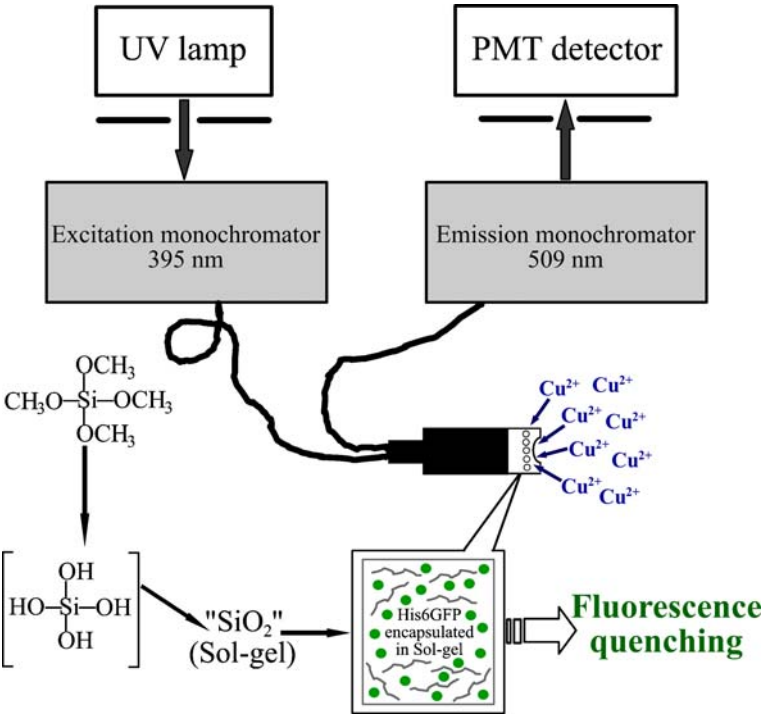

Fig. 7 Schematic representation of encapsulated His6GFP in Sol-gel immobilized on optical fiber connected with fluorescence measurement unit. This optical unit was doped into the metal solution, and the fluorescence response was instantaneously detected

Phen Green [33], Calcein [34], Fura-2 [35]) provide a high feasibility of using the encapsulated His6GFP in Sol-gel as a powerful tool for copper determination. Even though the detection limit to copper ions of the His6GFP is lower than those using the red fluorescent protein [13], however, the high expression yield as a monomeric form and the faster maturation rate of the His6GFP provide more attractive characteristics than the

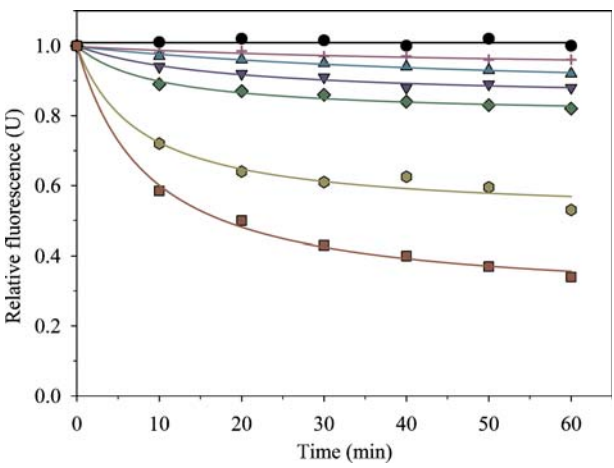

Fig. 8 Effect of various concentrations of copper ions on the fluorescence of encapsulated His6GFP in Sol-gel (un-optimized condition of TMOS). *Symbols* represent copper ions at 0 mM (*circle*), 0.5 μM (*plus sign*), 5 μM (*triangle*), 50 μM (*inverted triangle*), 0.5 mM (*diamond*), 5 mM (*hexagon*), and 50 mM (*square*)

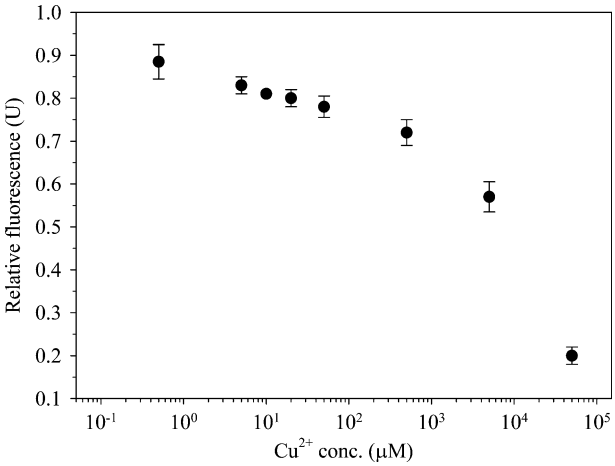

Fig. 9 Dose–response curve of His6GFP against various concentrations of copper ions. Data represent as mean ± SD (n=3)

others, especially for the encapsulation processes [36, 37]. Moreover, selection of the filter sets suitable for the shorter excitation and emission wavelength maxima of GFP can effectively be managed for sensor development.

Conclusion

Herein, a reagentless sensing unit based on exploiting the metal-mediated fluorescence quenching of GFP has successfully been developed for quantitation of copper ions. Real-time monitoring of fluorescence reduction of His6GFP encapsulated in Sol-gel reveals a high sensitivity and selectivity against copper ions in the range of 0.5 μM to 50 mM. Even though the limit of detection is approximately 3–6 orders of magnitude lower than those of atomic absorption spectroscopy and inductively coupled plasma mass spectroscopy. However, as a matter of fact, such sensitivity is sufficient to cover the range of toxic dose of copper ions (31 μM or 2 mg Cu/l) recommended by the World Health Organization

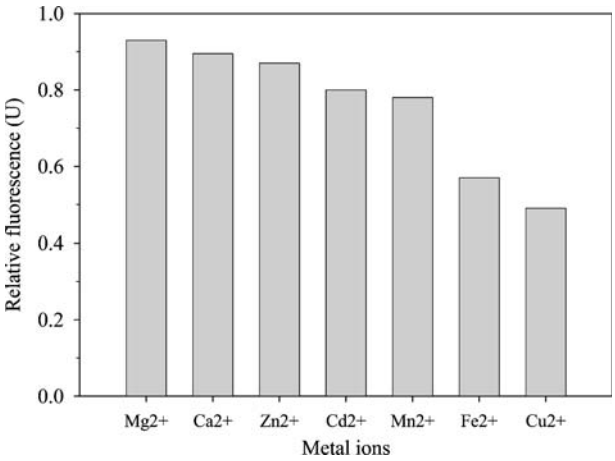

Fig. 10 Effects of various kinds of divalent cation (at 5 mM) on fluorescent emission of encapsulated His6GFP in Sol-gel

[2]. Moreover, the simplicity of handling, high stability, and effective reusability enlarge the application of fluorescent protein as a versatile tool for metal monitoring.

Acknowledgments This project was supported in part by the Young Scholars Research Fellowship from The Thailand Research Fund to T.T. (grant no. MRG5080158), the Deutsche Forschungsgemeinschaft (DFG) and the Bundesministerium fur wirtschaftliche Zusammenarbeit und Entwicklung (Federal Ministry for Economic Cooperation and Development; BMZ; grant no.GA233/19–1,2) and the annual budget grant of Mahidol University (B.E. 2551-2555). The authors would like to thank Prof. Dr. Hans-Ulrich Humpf (Institut für Lebensmittelchemie, Westfälische Wilhelms Universität) for his assistance on the operation of CD measurements.

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