A new water-soluble fluorescent Cu(II) chemosensor based on tetrapeptide histidyl-glycyl-glycyl-glycine (HGGG)

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A new water-soluble fluorescent chemosensor Fluor-HGGG was synthesized by linking a tetrapeptide histidyl-glycyl-glycyl-glycine (HGGG) with fluorescein via standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Significant fluorescence quenching was observed with Fluor-HGGG in the presence of Cu²⁺. Other metal ions including Ag⁺, Ca²⁺, Cd²⁺, Co²⁺, Fe²⁺, Fe³⁺, Hg²⁺, Mn²⁺, Ni²⁺ and Zn²⁺ produced only minor changes in fluorescence values for the system. The dissociation constant (K_D) of Cu²⁺ binding in Fluor-HGGG was found to be 37 μM. The maximum fluorescence quenching caused by Cu²⁺ binding in Fluor-HGGG was observed over the pH range 7–7.5.

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1. Introduction

Ionic copper is the third most abundant of the essential transition metal ions in the human body, and plays an important physiological role in many biological systems [1,2]. Due to its widespread applications, copper also represents a significant metal pollutant. Copper ions can react with molecular oxygen to form reactive oxygen species (ROS) capable of damaging proteins, nucleic acids and lipids. The cellular toxicity of ionic copper has been connected with serious neurodegenerative diseases including Menkes and Wilson diseases [3,4], Alzheimer’s disease [5] and prion disease [6]. The demand for more sensitive and selective Cu²⁺ detection both in vivo and in vitro is growing [7].

A general strategy used in developing metal ion chemosensors is to combine a metal-binding unit with signaling units such as fluorophores or chromophores. The presence of metal ions is signaled, during interaction with binding units, by changes in emission intensity or wavelength. A number of currently existing chemosensors consist of organic fluorophores or chromophores, which are undesirably insoluble in an aqueous solution. In order to resolve this solubility issue – a major obstacle in the fabrication of water-soluble metal ion chemosensors – the development of a suitable water-soluble metal-binding or signaling unit is critical. A few Cu²⁺ chemosensors based on peptides, GGH and GHK, have been developed to detect Cu²⁺ ions in aqueous system [7b,7c,7d,7e]. Tetrapeptide histidyl-glycyl-glycyl-glycine (HGGG) is a Cu²⁺ binding motif found in prion proteins (PrP) which displays highly selective binding toward Cu²⁺ [8,9]. According to a single crystal X-ray diffraction study of the HGGG-Cu²⁺ complex, Cu²⁺ binding in the complex was a tetradentate binding structure that involves the histidine imidazole, two deprotonated amides, and a glycine carbonyl [9]. Despite extensive research into the biological properties of tetrapeptide HGGG, no work has been done to investigate its potential as a chemosensor for Cu²⁺ detection. Here, a new water-soluble Cu²⁺ chemosensor Fluor-HGGG based on a tetrapeptide histidyl-glycyl-glycyl-glycine (HGGG) has been developed for Cu²⁺ sensing (Scheme 1). The HGGG motif was bound with fluorescein through an N-terminal amide bond; crucially, this does not inhibit the tetrapeptide HGGG binding to Cu²⁺ ions required in chemosensing applications. Fluorescein is one of the most powerful fluorescent probes known, due mainly to its high molar absorptivity and fluorescence quantum yield [10]. Metal ions such as Ag⁺, Ca²⁺, Cd²⁺, Co²⁺, Fe²⁺, Fe³⁺, Hg²⁺, Mn²⁺, Ni²⁺ and Zn²⁺ were tested for metal ion binding selectivity with Fluor-HGGG; Cu²⁺ was the only ion resulting in significant fluorescent quenching.

2. Experimental

2.1. Materials and instrumentations

N,N-dimethylformamide (DMF), Fmoc-Gly-Wang resin, Fmoc-Gly and Fmoc-His(Trt), 1-methyl-2-pyrrolidone (NMP) were purchased from Merck. N-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyllumonium...
of 20 mM HEPES buffer (pH 7.4) at 25°C. Fluorescence titration studies were conducted using an excitation wavelength of 490 nm and a maximum emission wavelength of 520 nm. Dissociation constant (K_d) was determined from analysis of the fluorescence quenching measurements [11]. Fluorescence quenching of fluorescein induced by the binding Cu(II) ions was used to calculate the fraction of binding sites occupied, f_b:

\[ f_b = \frac{y_b - y_s}{y_b - y_f} \]  

(1)

where y is the emission intensity at a given concentration of Cu(II) ions and y_b and y_s are the intensities when the binding sites are fully occupied and unoccupied, respectively. The binding function r is defined by Eq. (2) and p is the binding stoichiometry. The molar ratio of copper to Fluor-HGGG was 1:1 and the binding stoichiometry (p) was defined as 1. The dissociation constant (K_d) was determined by a fitting procedure from a plot of binding function r versus the concentration of Cu^{2+} (C_s) according to Eq. (3):

\[ r = \frac{pC_s}{(K_d + C_s)} \]  

(3)

In Fig. 5, the plot of binding function r versus the concentration of Cu^{2+} was fitted according to Eq. (3) and the dissociation constant (K_d) of Cu^{2+} binding in Fluor-HGGG was determined as 37 μM.

3. Results and discussion

3.1. Synthesis of Fluor-HGGG

The procedure for the synthesis of Fluor-HGGG was shown in Scheme 1. First, Fmoc-Gly-Wang resin was used as a solid support, and amino acid derivatives Fmoc–Gly and Fmoc–His(Trt) were attached stepwise through coupling reactions. The 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-hydroxybenzotriazole (HOBt) in situ activation method was used to characterize the excitation wavelength for fluorescence spectroscopy. Upon addition of Cu^{2+} (up to 10^{-3} M) to a solution containing chemosensor Fluor-HGGG, no significant absorption change at 490 nm was observed. The ligated Cu^{2+}–HGGG complex has a d–d transition band centered at 588 nm ([ε_{588} = 98 M^{-1} cm^{-1}]) [12]. In contrast to the strong absorption band of fluorescein complex only results in a minor change in absorbance at 588 nm; there is no obvious change in visible absorption.

To evaluate the selectivity of Fluor-HGGG toward various metal ions, the fluorescence spectra of Fluor-HGGG were taken in the presence of several transition metal ions. Fig. 1 shows the emission spectra of Fluor-HGGG under combination with Mn^{2+}, Fe^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}. The concentration of metal ions was 100 μM – 100-fold higher than the concentration of Fluor-HGGG.
(1.0 μM). Only Cu²⁺ induced significant fluorescence quenching. The mixture of Fluor-HGGG with Ca²⁺, Mg²⁺ and Hg²⁺ also induced minor variations in fluorescence relative to Fluor-HGGG. A plot of the ratio $F_{\text{Me}^2+}/F_{\text{metal free}}$ at 520 nm is shown in Fig. 2; most metal ions produced a ratio close to 1, the notable exception being Cu²⁺ with a low ratio of 0.1. These observations indicate that Cu²⁺ is the only ion readily bound with Fluor-HGGG to induce significant fluorescence quenching, permitting highly selective detection of Cu²⁺. Competitive experiments were carried out in the presence of Cu²⁺ (100 μM) with Ag⁺, Ca²⁺, Cd²⁺, Co²⁺, Fe²⁺, Fe³⁺, Hg²⁺, Mn²⁺, Ni²⁺ and Zn²⁺ at 100 μM (Fig. 3). Fluorescence quenching caused by the mixture of Cu²⁺ with the other metal ion was similar to that caused by only Cu²⁺. This indicates that other metal ions did not interfere the binding of Fluor-HGGG with Cu²⁺. This finding is consistent with previous studies suggesting that Cu²⁺ is the only metal ion that can be bound in the tetrapeptide HGGG [8].

### 3.3. Fluorescence titration analysis of Cu²⁺ in Fluor-HGGG

Emission spectroscopy of fluorescein revealed Cu²⁺ binding with Fluor-HGGG to be saturable. Various quantities of Cu²⁺ were incubated with Fluor-HGGG (1.0 μM in 20 mM HEPES buffer, pH 7.4), and emission spectra were recorded for each sample. Fig. 4 shows the plot of the emission intensity at 520 nm as a function of $-\log[\text{Cu}^{2+}]$; when Cu²⁺ concentration was higher than 100 μM, the fluorescence quenching reached a plateau. Half-maximal binding was reached at $5 \times 10^{-5}$ M Cu²⁺. Similar observations were made in a study of Cu²⁺ binding in prion proteins. Prion proteins contain four octarepeats (PHGGGWGQ) at the N-terminal, with a suitable fluorescent probe for Cu²⁺ binding found in tryptophan [8]. When Cu²⁺ was added in concentrations at least an order of magnitude higher than the prion protein solution (1.0 μM), significant fluorescence quenching was observed. Half-maximal binding in the prion protein was reached at $1.4 \times 10^{-5}$ M close to the $5 \times 10^{-5}$ M determined for Fluor-HGGG. The four octarepeats in prion proteins can bind Cu²⁺ cooperatively, thus lowering the half-maximal binding concentration relative to Fluor-HGGG. The detection limit of Fluor-HGGG as a fluorescent sensor for the analysis of Cu²⁺ was determined from the plot of fluorescence intensity as a function of the concentration of Cu²⁺ (see supplementary material). It was found that Fluor-HGGG has a detection limit of 3.9 μM, which is allowed for the detection of micromolar concentration range of Cu²⁺.

The dissociation constant ($K_D$) of Cu²⁺ binding with Fluor-HGGG was obtained from analysis of fluorescence quenching measure-
Fig. 5. Binding curve was generated from analysis of fluorescence quenching measurements and fitted in accordance with Eq. (2). The dissociation constant ($K_D$) of $Cu^{2+}$ in Fluor-HGGG was found to be 37 μM.

Fig. 6. Influence of pH on the fluorescence spectra for Fluor-HGGG (1.0 μM) both when pure and in combination with $Cu^{2+}$ (100 μM). Buffer: pH 5–7, 20 mM Mes buffer; pH 7–10, 20 mM HEPES buffer. The excitation wavelength was 490 nm, and the monitored emission wavelength was 520 nm.

3.4. Influence of pH on $Cu^{2+}$ binding in Fluor-HGGG

To investigate the pH range in which Fluor-HGGG can effectively detect $Cu^{2+}$, pH titration of Fluor-HGGG was first carried out. As depicted in Fig. 6, emission intensity increased over a pH range of 5–8 before reaching a plateau. Fluorescein exists in four ionic forms—cationic, neutral, mono-anionic, and di-anionic—each associated with distinct $pK_a$ values of 2.08, 4.31, and 6.43 [10]. Only the mono-anion and di-anion forms of fluorescein are capable of emitting fluorescence at quantum yields 0.37 and 0.93, respectively [10]. At pH > 6.5, the di-anion form is dominant and the emission intensity is higher relative to the mono-anion form [10]. This accounts for the significant increase in fluorescence when the system pH is 6.5—close to the $pK_a$ value of the di-anion form. At pH < 6.5, the mono-anion form is dominant and an associated drop in emission intensity is observed. At pH < 5 the fluorescence intensity is very low, and data over this range is not illustrated in Fig. 6. The pH profile of Fluor-HGGG is identical to that of fluorescein.

The effect of $Cu^{2+}$ binding in Fluor-HGGG was pronounced when considering emission intensity response to changes in system pH, with metal-free Fluor-HGGG species exhibiting substantially different behavior. In Fig. 5 addition of $Cu^{2+}$ is shown to cause significant fluorescence quenching for pH > 6.5, reaching a maximum in the pH range 7–7.4. For pH values exceeding 8, emission intensity was maximized and reached a stable plateau. This titration curve indicates the optimal pH range for fluorescence quenching to be between 7 and 8, which is a physiologically viable cell pH. According to a study of $Cu^{2+}$ binding in the HGGG motif using potentiometric measurements [12], $CuH_{2}L$ (L, Fluor-HGGG) is a dominant species for pH values ranging from 6 to 9 (Scheme 2). Histidine imidazole is suggested as the primary $Cu^{2+}$ binding site, followed by deprotonation at two of the amide bonds; deprotonation of histidine imidazole is required for $Cu^{2+}$ binding. This accounts for significant fluorescence quenching at pH values above 6.5—close to the $pK_a$ value of histidine imidazole, 6.0. When the pH values were higher than 8, the emission intensities were slightly higher than that at pH 7.5 (Fig. 6). The slightly higher emission intensities observed for pH values above 8 are due to the formation of a new species, $CuH_{3}L$ (L, Fluor-HGGG). The species, $CuH_{3}L$, is formed at pH values greater than 8 by deprotonation of the third amide, and comes to dominate for pH values > 9. $Cu^{2+}$ binding in both species, $CuH_{2}L$ and $CuH_{3}L$, caused significant fluorescent quenching. Differently, the species,
CuH$_{3}$L, has slightly higher emission intensities than the species, CuH$_{2}$L. This accounts for minor change in emission intensities at pH 8.

4. Conclusions

We have developed a Cu$^{2+}$ chemosensor Fluor-HGGG that was synthesized by linking a tetrapeptide histidyl-glycyl-glycyl-glycine (HGGG) with a fluorescein via standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The recognition of Cu$^{2+}$ ion by Fluor-HGGG gave rise to significant fluorescence quenching; addition of Ag$^{+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Hg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$ or Zn$^{2+}$ to the chemosensor solution caused only minimal change in fluorescence emission values. This peptide based Cu$^{2+}$ chemosensor should provide an effective means of Cu$^{2+}$ sensing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.snb.2009.05.035.

References


Biography

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