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

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ABSTRACT

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 Cu^{2+}. Other metal ions including Ag^{+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} produced only minor changes in fluorescence values for the system. The dissociation constant (K_D) of Cu^{2+} binding in Fluor-HGGG was found to be 37 μM. The maximum fluorescence quenching caused by Cu^{2+} binding in Fluor-HGGG was observed over the pH range 7–7.5.

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 Cu^{2+} 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 Cu^{2+} chemosensors based on peptides, GGH and GHK, have been developed to detect Cu^{2+} ions in aqueous system [7b,7c,7d,7e].

Tetrapeptide histidyl-glycyl-glycyl-glycine (HGGG) is a Cu^{2+} binding motif found in prion proteins (PrP) which displays highly selective binding toward Cu^{2+} [8,9]. According to a single crystal X-ray diffraction study of the HGGG-Cu^{2+} complex, Cu^{2+} 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 Cu^{2+} detection. Here, a new water-soluble Cu^{2+} chemosensor Fluor-HGGG based on a tetrapeptide histidyl-glycyl-glycyl-glycine (HGGG) has been developed for Cu^{2+} sensing (Scheme 1). The HGGG motif was bound with fluorecein through an N-terminal amide bond; crucially, this does not inhibit the tetrapeptide HGGG binding to Cu^{2+} 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^{+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} were tested for metal ion binding selectivity with Fluor-HGGG; Cu^{2+} 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- tetramethyluronium

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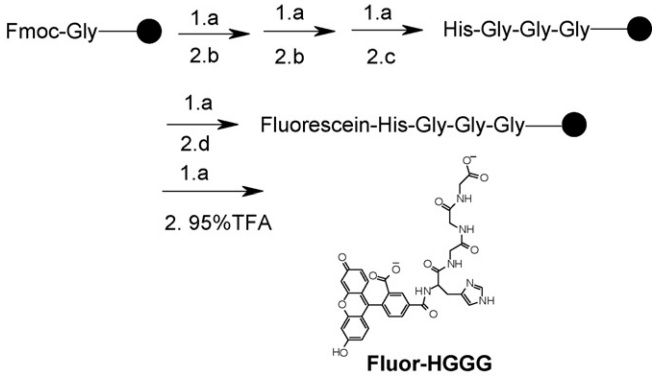

Scheme 1. Synthesis of Fluor-HGGG. (a) 22% piperidine in NMP (b) Fmoc-Gly-OH, HBTU, HOBt, NMP in DMF (c) Fmoc-L-His(Trt)-OH, HBTU, HOBt, NMP in DMF. (d) 6-Carboxyfluorescein, HBTU, HOBt, NMP in DMF.

hexafluorophosphate (HBTU) were purchased from Applied Biosystem. $AgClO_4$, $Cd(ClO_4)_2.xH_2O$, $Cu(BF_4)_2$, $CoCl_2{\cdot}6H_2O$, $FeCl_3$, $Fe(BF_4)_2{\cdot}6H_2O$, $Hg(ClO_4)_2{\cdot}xH_2O$, $MnSO_4{\cdot}H_2O$, $Ni(CH_3COO)_2{\cdot}4H_2O$, $Zn(BF_4)_2$, trifluoroacetic acid were purchased from Sigma–Aldrich. $CaCO_3$ and $MgSO_4$ were purchased from Showa. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Bio Basic Inc. 2-(*N*-Morpholine)-ethane sulphonic acid (MES) was purchased from Amresco Inc. UV–vis spectra were recorded on an Agilent 8453 UV–vis spectrophotometer. Fluorescence spectra were recorded in a Hitachi F-4500 spectrometer. Peptide synthesis was done by Applied Biosystems ABI 433A Peptide Synthesizer.

2.2. Synthesis of Fluor-HGGG

Synthesis of Fluor-HGGG was via standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. During the synthesis, Fmoc-Gly-Wang resin was used as a solid support and amino acid derivatives, Fmoc-Gly and Fmoc-His(Trt), were attached step by step through coupling reaction. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) in situ activation method was used for the coupling reactions and deprotecting Fmoc group was done with piperidine. 6-Carboxyfluorescein was finally coupled to the N-terminal of tetrapeptide HGGG. Deprotection of trityl group (Trt) from histidine and cleavage of Fluorescein-His-Gly-Gly-Gly from the resin were accomplished by trifluoroacetic acid (TFA). Crude peptide was furthermore purified by HPLC (C18 column) and purified peptide was confirmed by ESI-Mass. The formula of Fluor-HGGG was $C_{33}H_{26}N_6O_{11}$ with molecular weight (calculated) 682.182, ESI-mass (measured) 682.222.

2.3. Metal ion binding study by UV–vis and fluorescence spectroscopy

Fluor-HGGG (1.0 μM) was added with different metal ions (100 μM). All spectra were measured in 1.0 mL of 20 mM HEPES buffer (pH 7.4) at 25 °C. The light path length of cuvette was 1.0 cm. The excitation wavelength was 490 nm and the maximum emission wavelength was 520 nm.

2.4. Fluorescence titration studies

Fluor-HGGG (1.0 μM) was added with different concentration of Cu^{2+} (10^{-7} to 10^{-3} μM). All spectra were measured in 1.0 mL of 20 mM HEPES buffer (pH 7.4) at 25 °C. The light path length of cuvette was 1.0 cm. The excitation wavelength was 490 nm and the maximum emission wavelength was 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_a:

$$f_a = \left(\frac{y - y_f}{y_b - y_f}\right) \quad (1)$$

where y is the emission intensity at a given concentration of Cu(II) ions and y_b and y_f 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):

$$r = f_a * p \quad (2)$$

$$r = \frac{pC_s}{(K_D + C_s)} \quad (3)$$

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 for coupling, and piperidine for Fmoc deprotection. Finally, 6-carboxyfluorescein was attached to the N-terminal of tetrapeptide HGGG before deprotection of the trityl group (Trt) from histidine and cleavage of fluorescein-His-Gly-Gly-Gly from the resin by treatment with trifluoroacetic acid (TFA). The crude peptide product was purified by High Pressure Liquid Chromatography (Angilent ZORBAX 300sb-C18, 9.4 × 250 mm), and the separated Fluor-HGGG component was identified by ESI-mass spectroscopy.

3.2. Cu(II) sensing by Fluor-HGGG

The absorption spectrum of Fluor-HGGG exhibited three maximum bands at wavelengths 210, 240 and 490 nm (pH = 7.4). The absorption band centered at 490 nm results from fluorescein with a high extinction coefficient (ε_{490} = 76,900 M^{-1} cm^{-1}), and is thus used to characterize the excitation wavelength for fluorescence emission [10]. Upon addition of Cu^{2+} (up to 10^{-4} 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 compounds at 490 nm, formation of the Fluor-HGGG-Cu^{2+} 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