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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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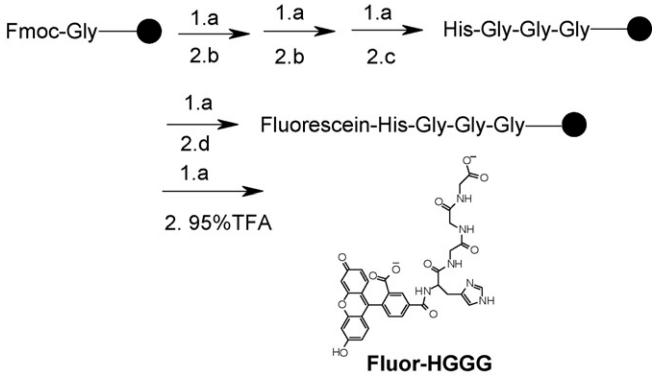


Scheme 1. Synthesis of Fluor-HGGG. (a) 22% piperidine in NMP (b) Fmoc-Gly-OH, HBTU, HOBt, NMP in DMF (c) Fmoc-L-His(Trt)-OH, HBTU, HOBt, NMP in DMF. (d) 6-Carboxyfluorescein, HBTU, HOBt, NMP in DMF.

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

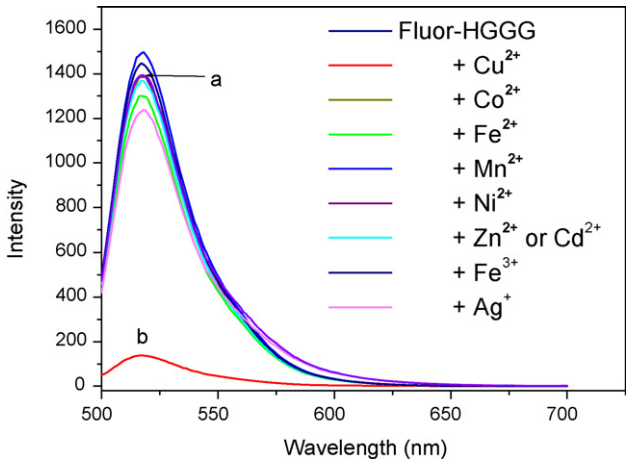


Fig. 1. . Response of chemosensor Fluor-HGGG to different metal ions in terms of fluorescence. Curve a shows the fluorescence spectrum of metal-free Fluor-HGGG (1.0 μM), with maximum emission wavelength 520 nm. Curve b represents the spectrum after addition of Cu^{2+}. The concentration of each metal ion was 100 μM. All spectra were taken at 25 °C in 20 mM HEPES buffer (pH 7.4) at excitation wavelength 490 nm.

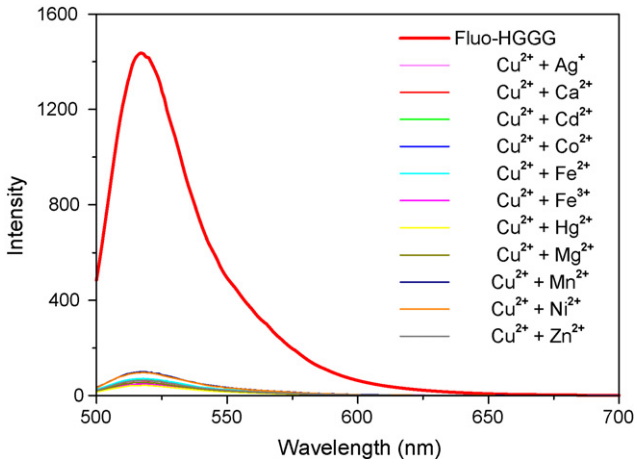


Fig. 3. Fluorescent response of Fluor-HGGG (1.0 μM) to Cu^{2+} (100 μM) over the selected metal ions (100 μM). All spectra were taken at 25 °C in 20 mM HEPES buffer (pH 7.4) at excitation wavelength 490 nm.

(1.0 μM). Only Cu^{2+} induced significant fluorescence quenching. The mixture of Fluor-HGGG with Ca^{2+}, Mg^{2+} and Hg^{2+} also induced minor variations in fluorescence relative to Fluor-HGGG. A plot of the ratio $F_{M(II)}/F_{metal\,free}$ at 520 nm is shown in Fig. 2; most metal ions produced a ratio close to 1, the notable exception being Cu^{2+} with a low ratio of 0.1. These observations indicate that Cu^{2+} is the only ion readily bound with Fluor-HGGG to induce significant fluorescence quenching, permitting highly selective detection of Cu^{2+}. Competitive experiments were carried out in the presence of Cu^{2+} (100 μM) with Ag^{+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} at 100 μM (Fig. 3). Fluorescence quenching caused by the mixture of Cu^{2+} with the other metal ion was similar to that caused by only Cu^{2+}. This indicates that other metal ions did not interfere the binding of Fluor-HGGG with Cu^{2+}. This finding is consistent with previous studies suggesting that Cu^{2+} is the only metal ion that can be bound in the tetrapeptide HGGG [8].

3.3. *Fluorescence titration analysis of Cu^{2+} in Fluor-HGGG*

Emission spectroscopy of fluorescein revealed Cu^{2+} binding with Fluor-HGGG to be saturable. Various quantities of Cu^{2+} were incubated with Fluor-HGGG (1.0 μM in 20 mM HEPES buffer, pH = 7.4),

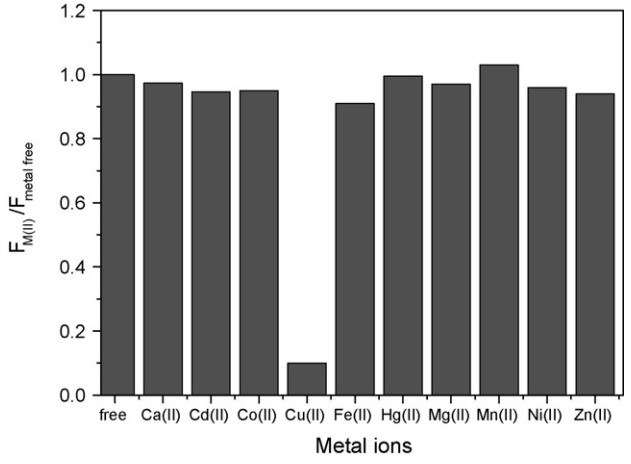


Fig. 2. Emission intensity ratios ($F_{metal\,ion}/F_{metal\,free}$) for chemosensor Fluor-HGGG at 520 nm in the presence of different metal ions.

and emission spectra were recorded for each sample. Fig. 4 shows the plot of the emission intensity at 520 nm as a function of $-\log[Cu^{2+}]$; when Cu^{2+} concentration was higher than 100 μM, the fluorescence quenching reached a plateau. Half-maximal binding was reached at 5×10^{-5} M Cu^{2+}. Similar observations were made in a study of Cu^{2+} binding in prion proteins. Prion proteins contain four octarepeats (PHGGGWGQ) at the N-terminal, with a suitable fluorescent probe for Cu^{2+} binding found in tryptophan [8]. When Cu^{2+} 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 1.4×10^{-5} M – close to the 5×10^{-5} M determined for Fluor-HGGG. The four octarepeats in prion proteins can bind Cu^{2+} 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 Cu^{2+} was determined from the plot of fluorescence intensity as a function of the concentration of Cu^{2+} (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 Cu^{2+}.

The dissociation constant (K_D) of Cu^{2+} binding with Fluor-HGGG was obtained from analysis of fluorescence quenching measure-

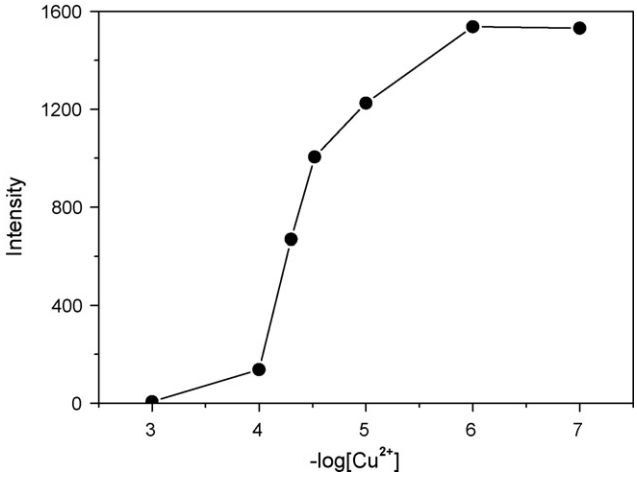


Fig. 4. Titration curve of fluorescence emission intensity for Fluor-HGGG against the negative log of Cu^{2+} concentration. The concentration of Fluor-HGGG was 1.0 μM in 20 mM HEPES buffer (pH 7.4). Excitation wavelength was 490 nm, and the monitored emission wavelength was 520 nm.

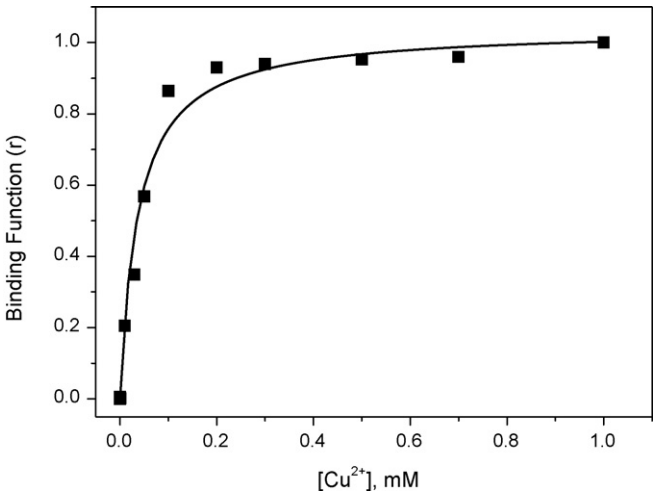


Fig. 5. Binding curve was generated from analysis of fluorescence quenching measurements and fitted in accordance with Eq. (2). The dissociation constant (K_D) of Cu^{2+} in Fluor-HGGG was found to be 37 μM.

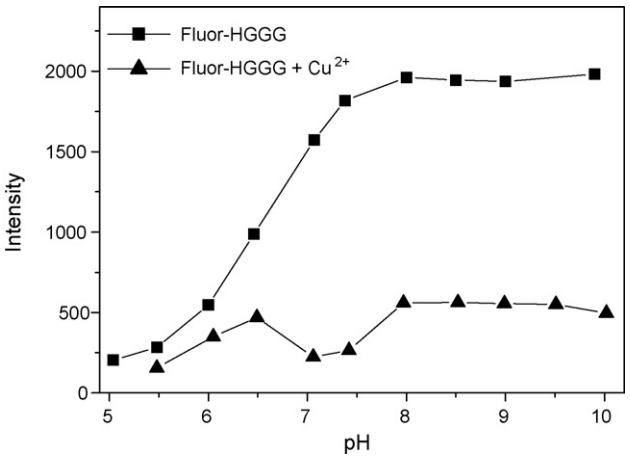


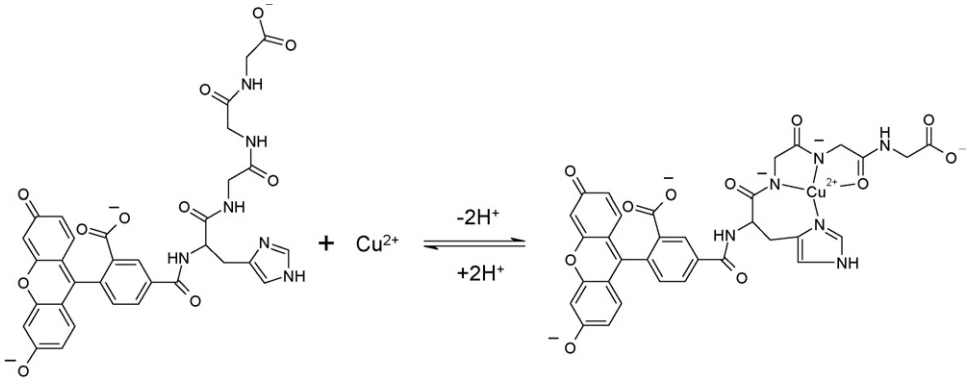
Fig. 6. Influence of pH on the fluorescence spectra for Fluor-HGGG (1.0 μM) both when pure and in combination with Cu^{2+} (100 μM). Buffer: pH 5 ~7, 20 mM Mes buffer; pH 7–10, 20 mM HEPES buffer. The excitation wavelength was 490 nm, and the monitored emission wavelength was 520 nm.

ments. The degree of fluorescein fluorescence quenching induced by the binding Cu^{2+} ions was used to calculate the fraction of binding sites occupied. The binding function, *r*, is defined by Eq. (2) where *p* is the binding stoichiometry. The dissociation constant was determined by fitting a plot of binding function *r* versus the concentration of Cu^{2+} (C_S) in accordance with Eq. (3). In Fig. 5, a plot of binding function against the concentration of Cu^{2+} was fitted according to Eq. (3), and the dissociation constant of Cu^{2+} binding with Fluor-HGGG was found to be 37 μM.

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,



Scheme 2. Molecular structure of Cu^{2+}–Fluor-HGGG.

$CuH_{-3}L$, 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.

Acknowledgments

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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.

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