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A New Fluorescent Chemosensor for Copper Ions Based on Tripeptide Glycyl–Histidyl–Lysine (GHK)

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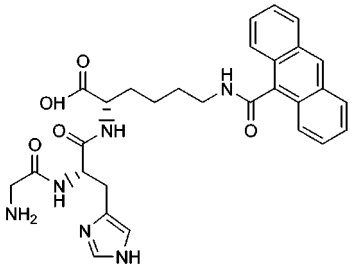
ABSTRACT



A new fluorescent chemosensor for Cu^{2+} ions was synthesized by modifying the tripeptide glycyl–histidyl–lysine (GHK) with 9-carbonylanthracene via the standard Fmoc solid-phase peptide synthesis method. While significant fluorescence quenching was observed from the molecule upon binding with Cu^{2+}, addition of Fe^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} to the peptide solution caused a minimum fluorescence emission spectral change, indicating a high specificity of this chemosensor for Cu^{2+} ions. Effects of pH were also investigated.

The development of fluorescent molecular sensors for metal ions, especially for cations with biological interest such as Na^+, Ca^{2+}, Cu^{2+}, and Zn^{2+}, has always been of particular interest.[1] One strategy employed in the design of chemosensors for metal cations is to link a fluorophore unit with a metal binding unit (ionophore). The two units are linked to each other in such a way that the binding of a cation to the ionophore causes considerable changes in the fluorescence of the fluorophore. Such changes can be intensity, intensity ratio, anisotropy, time-domain lifetime or phase-modulation lifetime, etc.[1e,2]

The high sensitivity and abundance of fluorophores makes fluorescence technique among one of the most promising tools for chemo- and biosensor development. However, the selectivity of fluorescent chemosensors for metal cations remains a significant challenge. A number of currently available fluorescent probes for metal cations actually change

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their fluorescent properties upon binding with more than one metal cations.[1e] For example, Fura-2 is a fluorescent probe for Ca^{2+} but also binds with Zn^{2+} with considerable high affinity. Mg^{2+} fluorescence indicators such as Mag-Fura-2, Mag-Fura-5, and Mag-Indo-1 all bind with Ca^{2+}, although with low affinity.

Copper is third in abundance (after Fe^{3+} and Zn^{2+}) among the essential heavy metals in the human body and plays an important role in various physiologic processes. The design and synthesis of chemosensors for copper ions has become a very active area of research as a result of the demand for more sensitive and selective chemosensors for in vitro and in vivo purposes.[3] Recently, combinatorial library technique was employed to identify peptide or peptidomimetic ligand structures with specific Cu^{2+} binding activities.[4] Lead molecules discovered through library screening were linked with a fluorescent transducer, and the selectivity of these sensor molecules toward Cu^{2+} was largely increased.

Tripeptide growth factor glycyl−L-histidyl−L-lysine (GHK) is a human plasma constituent that is well-known for its activity to promote cell growth and wound healing.[5] Single-crystal X-ray diffraction study of the GHK−Cu^{2+} complex revealed a monomeric tridendate binding structure that involves the N-terminal group of glycine, the nitrogen atom of the first amide bond, the unprotonated imidazole nitrogen from histidine, and two oxygen atoms in water.[6] The lysyl side chain amino group does not participate in the binding with copper but is involved in cell recognition and interaction.[6c]

Despite its well-studied biological function and activity, it appeared to us that no work has been reported on the modification of GHK with fluorophores as a potential fluorescent chemosensor for Cu^{2+}. On the basis of the structural motif of the GHK−Cu^{2+} complex, we reasoned that the modification of the lysyl side chain amine by a fluorophore might endow fluorescent characteristics to the resultant molecule and simultaneously retain the binding property of the tripeptide toward Cu^{2+} ions for sensing purposes, as shown in Figure 1. 9-Carbonylanthracene was

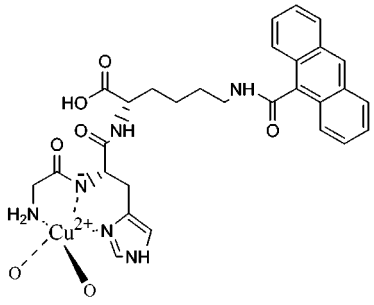
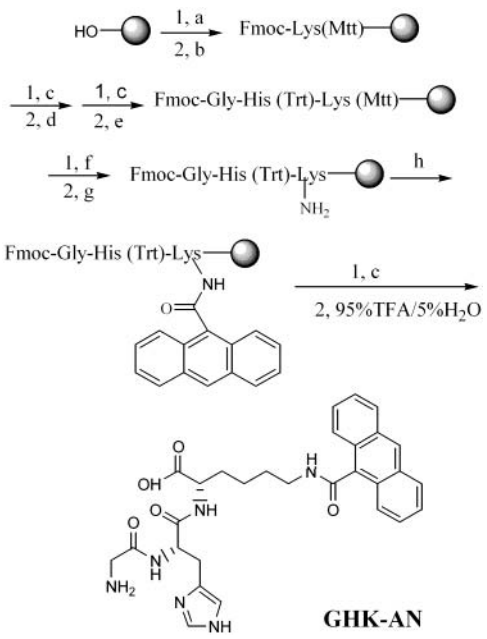


Figure 1. Proposed binding model of GHK-AN with Cu^{2+}.

used as the fluorophore unit. Anthracene derivatives may be excited with relatively short wavelengths (250−380 nm) to give emission bands around 400−500 nm, a blue emission band, which makes anthracene suitable for multicolor fluorescence applications when used together with long wavelength fluorescence probes such as fluorescein and rhodamines. Furthermore, the fluorescence of 9-carbonylanthracene moiety itself is not influenced by pH variations, which is an important advantage compared to pH-dependent fluorophores such as fluorescein. Since the ionophore here is a pH-dependent peptide, the use of a pH-dependent fluorescence probe will surely increase the complexity of the analysis results and should be avoided.

The 9-carbonylanthracene derivative of GHK (GHK-AN) was synthesized via standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Scheme 1).[7] Diisopropylcarbodiimide (DIC) and 1-hydroxylbenzotriazole (HOBt) in situ activation method was used for the coupling reactions. The

Scheme 1[a]



[a] (a) Fmoc-L-Lys(Mtt)-OH, DMAP, DIC, HOBt, 3 h. (b) Ac_2O and pyridine, 0.5 h. (c) 20% piperidine/DMF. (d) Fmoc-L-His(Trt)-OH, DIC, HOBt, 0.5 h. (e) Fmoc-Gly-OH, DIC, HOBt. 0.5 h. (f) 1% TFA/DCM, 0.5 h. (g) 10% TEA, 15 min. (h) 9-Anthracenecarboxylic acid, HOBt, DIC, 12 h.

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first amino acid lysine with 4-methyltrityl (Mtt) as the side chain protecting group was loaded to Wang resin according to the reported procedure.[8] After washing, drying, and deprotecting the Fmoc group with piperidine, histidine and glycine were sequentially coupled to the peptide. The Mtt protecting group was removed with 1% TFA in dichloromethane.[9] The 9-anthracenecarboxylic acid was then coupled with the deprotected lysine side amino group at the presence of activation reagents DIC and HOBt. At the end of the coupling reaction, the Fmoc group from N-terminal glycine was deprotected with 20% piperidine in DMF. The deprotection of trityl group (Trt) from histidine and cleavage of GHK-AN from the resin were accomplished with CF_3-COOH/H_2O (95/5, v/v) for 2.5 h. Following vacuum filtration and removal of TFA with N_2 blow-off, crude product was precipitated from cold ether. The solid precipitate was centrifuged, washed with ether, and lyophilized under vacuum. The crude product was further purified with semipreparative HPLC. Experimental details and characterizations of the product can be found in Supporting Information.

The absorption and emission spectra of GHK-AN are shown in Figure 2. Although the molecule has a clear

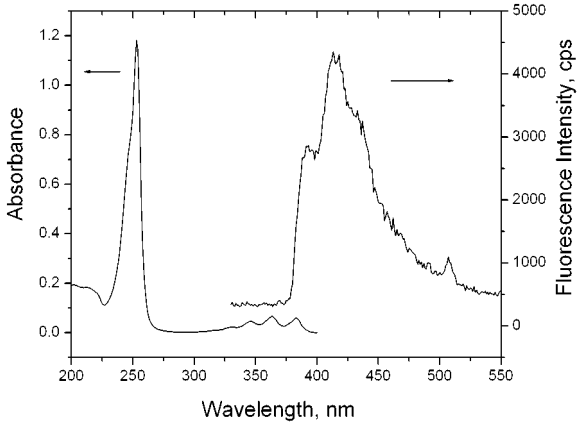

Figure 2. Absorption and emission spectra of GHK-AN in aqueous solution (PBS buffer, pH = 7.4). In the absorption spectrum, optical path length = 1 cm. ϵ_{253nm} = 118 188 L mol^{-1} cm^{-1}; ϵ_{364nm} = 6612 L mol^{-1} cm^{-1}. In the emission spectrum, excitation wavelength = 253 nm. The small band at 507 nm is the secondary harmonic emission from the excitation light.

absorption band at 364 nm, 253 nm was chosen for excitation to avoid the influence of Raman scattering of the aqueous solvent. A typical anthracene emission spectrum with a maximum emission band centered at 415 nm was observed at GHK-AN concentration of 10^{-6} M. Upon the addition of $CuCl_2$ to the solution, the fluorescence was greatly quenched. In addition, the appearance of an absorption band at 600 nm, in the absorption spectrum, further demonstrated the complexation of GHK-AN toward copper ions (data not shown).

A titration experiment was conducted to determine the binding ratio between Cu^{2+} and the GHK-AN molecule (Figure 3). From the titration curve of Cu^{2+} to an aqueous

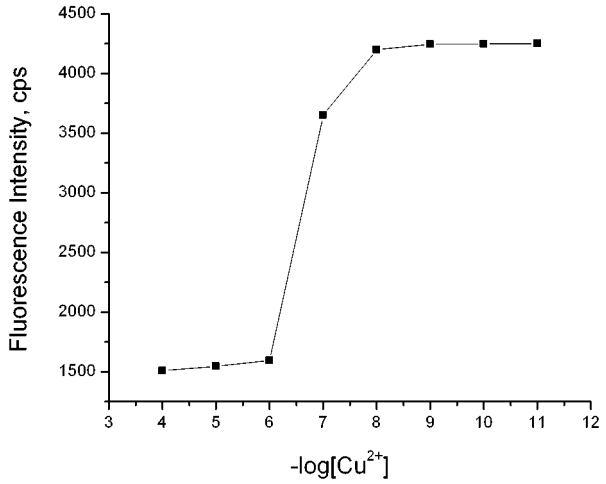

Figure 3. Titration curve of the fluorescence intensity (emission wavelength = 415 nm) of GHK-AN versus the concentration of Cu^{2+} (negative log value). The concentration of GHK-AN is kept at 10^{-6} M in PBS buffer, pH = 7.4. Excitation wavelength = 253 nm.

solution of GHK-AN (10^{-6} M), one can clearly see that a complexation at a molar ratio of 1:1 occurred between the GHK-AN and copper ions. The fluorescence of GHK-AN at 415 nm was quenched to 34% of its original intensity when the concentration of Cu^{2+} is equal to or greater than 10^{-6} M. We further determined the binding constant using a binding model reported in the literature.[10] According to this model, we obtained a binding constant of 10^{16} M^{-1} between Cu^{2+} and GHK-AN, which is essentially the same as that of GHK.[6a,6b] This indicates that the modification of GHK with 9-carbonylanthracene did not change the binding activity of the peptide toward Cu^{2+}.

We also determined the pH influence on the fluorescence of GHK-AN and its complex with Cu^{2+} (Figure 4). At pH higher than 5.5, GHK-AN is strongly fluorescent while the fluorescence of its complex with copper is largely quenched. However when pH is lower than 5.5, the fluorescence of GHK-AN−copper complex begins to revive, and at pH 4.5, it is almost the same as that of GHK-AN ligand. This corresponds to the dissociation of GHK-AN with Cu^{2+}. At pH ≤ 2.5, the fluorescence emission is largely quenched. This is probably due to the protonation of the C-terminal carboxyl group of lysine amino acid (pK_a 2.16). This indicates the importance of the carboxyl group to the fluorescence of the GHK-AN. The change of the carboxyl

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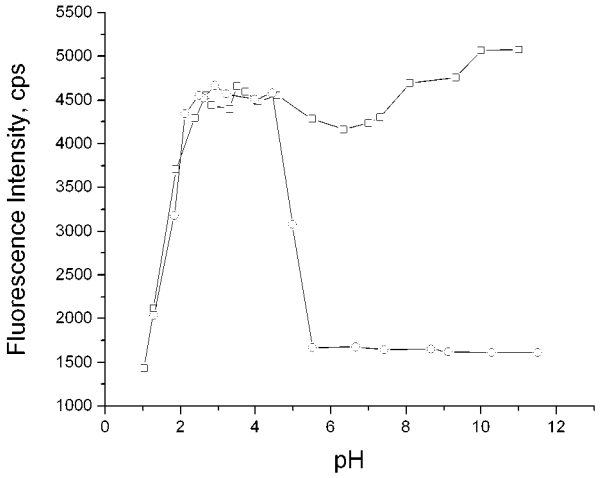

Figure 4. Influence of pH on the fluorescence of GHK-AN (10^{-6} M) (□) and its 1:1 mixture with Cu^{2+} (○). Excitation wavelength = 253 nm.

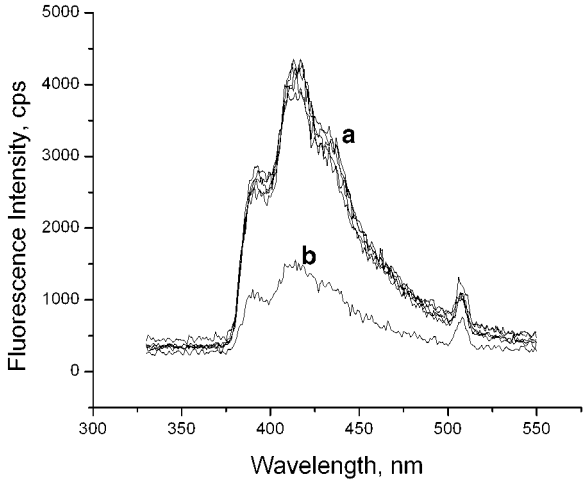

Figure 5. Fluorescence response of GHK-AN to different metal ions (excitation at 253 nm). Concentration of GHK-AN is kept constant at 10^{-6} M in aqueous solution. In curves **a**, it is shown that Fe^{2+}, Zn^{2+}, Co^{2}, and Ni^{2+} (0.1 mM, respectively) do not cause obvious changes in the fluorescence intensity of GHK-AN. Curve **b** shows that the fluorescence intensity of GHK-AN is greatly quenched by Cu^{2+} (10^{-6} M). PBS buffer (pH = 7.4).

group from a negatively charged state to neutral likely causes the conformational change of the whole peptide. More importantly, the GHK part is now highly protonated, and this electron-deficient nature may cause an AN*-to-GHK electron-transfer process, which is responsible for the observed fluorescence quenching. More detailed experiments will be appropriate in delineating these mechanisms.

To test the selectivity of the fluoroionophore for copper ion binding, the fluorescence responses of GHK-AN to several other transition metal salts including $FeCl_2$, $CoCl_2$, $NiCl_2$, and $ZnCl_2$ were measured and compared to that of copper ions (Figure 5). Here we can clearly see that even at a concentration as high as 0.1 mM, the presence of these transition metals caused little or no change to the fluorescence emission intensity of GHK-AN. On the other hand, in the presence of Cu^{2+} at a much lower concentration, i.e., 10^{-6} M, the fluorescence of GHK-AN was quenched down to 34% of its original intensity (emission wavelength = 415 nm).

Interestingly, it has been reported that the GHK associates with transition metal ions such as ionic copper, cobalt, iron, manganese, nickel and zinc and alters the patterns of cell growth in monolayer cultures of tumorigenic heptoma cell line (HTC4).[11] In our case, we showed that the fluorescence intensity response of GHK-AN toward the binding of these transition metal ions is only unique for copper. This may suggest that transition metal ions such as Fe^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} do not bind with GHK with same structural motif as Cu^{2+} does.

In conclusion, we designed a novel copper fluorescent chemosensor based on the tripeptide glycyl−histidyl−lysine. It shows a high specificity for copper ions at the physiological pH. Currently we are synthesizing a series of peptide lipids and using Langmuir and Langmir−Blodgett film techniques[12] to develop surface-oriented chemosensor devices for copper and other transition metal ions.

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Supporting Information Available: Experimental methods and characterizations by FAB, HPLC, and NMR. This material is available free of charge via the Internet at http://pubs.acs.org.

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