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The switch-on luminescence sensing of histidine-rich proteins in solution: a further application of a Cu^{2+} ligand†

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A new probe/Cu^{2+} complex for the detection of his-tagged protein has been developed, based on an improved probe, Dansyl-Gly-Py (1), by closely mimicing the structure of a peptide, ATCUN. In aqueous solution, 1/Cu^{2+} has good selectivity to histidine and cysteine, and further can detect histidine-rich protein by releasing the quenched fluorescence of 1.

The analysis and detection of protein in biological system is a crucial challenging area as traditionally which requires tools for purifying, handling, and/or modifying of the protein. Therefore, recently two very promising approaches have been developed. The first strategy is the specific site labeling by using fluorescent proteins (FPs) as green fluorescent protein,[1] quantum dots (QDs)[2] or small organic fluorescence probes.[3] The second one is related to the specific ligand in chelating with metal ions, to anchor the residues that could co-ordinate with the metal ion more strongly. For example, Song and co-workers[4] have developed a nickel-nitrilotriacetate-modified quantum dots which can image the protein by anchoring the His residues; Hamachi and coworkers[5] have developed a Oligo-Asp tag/Zn(II) complex which was used as a new pair for labeling and fluorescence imaging of proteins, where Cy3-modified zinc ligand can anchor the Asp residues by forming the co-ordinate complex. Recently, Wong[6] has developed a highly selective luminescent switch-on probe for the detection of histidine-rich proteins, in which once the histidine or histidine-rich proteins chelated with the iridium(III) complex the strong fluorescence emission will appear. Being inspired by this, we attempted to use the "reverse" strategy to detect the histidine-rich proteins. When histidine and histidine-rich proteins was added into a solution containing a fluorescent probe/metal ions complex, as the histindine would snatch the metal ions from the complex and the quenched fluorescence by metal ion will be released again.

In the previous work, we have developed a tetrafunctional probe based on peptide and dansyl motif, which responded to Hg^{2+} and Cu^{2+} in distinct modes with distinguishing optical properties.[7] After the fluorescence was quenched by Cu^{2+}, once we further add histidine or other amino acids the fluorescence of probe will be recovered (Fig. S1 and S2, ESI†). The results demonstrated that the general amino acid can snatch the Cu^{2+} from the reported probe/Cu^{2+} complex, which supplied a potential application of ligand in the detection of amino acids.

Although we have improved the water-solubility of probe at some extend, the selectivity to amino acids was still poor. As illustrated in Fig. S2 almost all the amino acids can snatch Cu^{2+} in the presence of probe because the stronger binding affinity between Cu^{2+} and amino acids (see ESI†). Therefore, further efforts would be paid to improve the binding force between Cu^{2+} and probe by optimizing the structure. As the ATCUN sequence has been proved to be the most strong Cu^{2+} binding structure,[8] the tryptophan was firstly employed to simulate the Cu^{2+} coordination structure. The previous dipeptide sensor, DGT,[7] containing the indole ring which was expected to form loop structure with Cu^{2+} through sulfonamide, amide and the nitrogen of indole ring, although actually the indole ring did not. In fact, generally the pyrrole-type nitrogen of indole could not chelate Cu^{2+} so efficiently as the pyridine-type nitrogen in imidazole.[9] Such limitation inspired us to design a new fluorescence probe, Dansyl-Gly-Py (**1**), for Cu^{2+} by introducing the amino methyl pyridine in mimicing the ATCUN structure more closely. To do so, we hoped the binding affinity of the new peptide probe to Cu^{2+} could be improved, and finally the selectivity of probe/Cu^{2+} complex to amino acids could be improved significantly.

The synthesis of **1** was started with free glycine (**2**), which was firstly phthaloylated with phthalic anhydride to give N-phthalimido-protected amino acid **3** in a high yield. The resulted pure **3** was coupled with amino methyl pyridine to give peptide analog **4** directly without further purification. Hydrazinolysis of the phthalimido protected **4** produced the crucial amine intermediate **5**, which was unstable to heat or prolonged storage at room temperature. Free amine **5** was immediately mixed with dansyl chloride in CH_2Cl_2 solution to give the molecule **1**. The final target, probe **1** was assayed with Mass, ^1H NMR and ^{13}C NMR spectroscopy, respectively (see ESI†).

Firstly, we explored the fluorescence response of **1** to various metal ions. Upon addition of metal ions into the buffer solution containing **1** (pH 7.4), similarly as the previous probe (DGT),[7] **1** responsed to Hg^{2+} and Cu^{2+} in distinct modes with distinguishing optical properties: it responded to Hg^{2+} with enhanced and blue-shifted fluorescence emission but to Cu^{2+} with unilateral fluorescence quenching (Fig. 1). Fitting the fluorescence changes of **1** with Cu^{2+} concentration, using the modified Stern–Volmer

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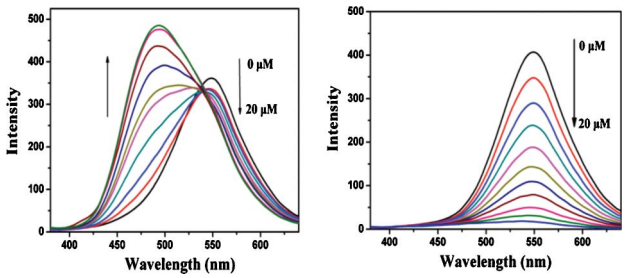

Fig. 1 Fluorescence spectra of **1** (20 μM) in HEPES buffer solution (pH = 7.4) in titrated with Hg^{2+} (left) and Cu^{2+} (right), respectively (λ_{ex} = 330 nm).

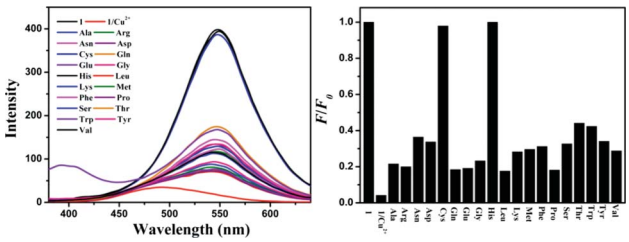

Fig. 3 The fluorescence intensity changes of **1**/Cu^{2+} (20: 20 μM) upon the addition of various amino acid in buffer solution, indicating that the fluorescence intensity of **1** can be released after the coordinated Cu^{2+} was extracted by amino acid.

equation,[10] gave a good linear correlation and demonstrated the formation of the complex with a 3.98×10^6 M^{-1} binding constant in 1 : 1 stoichiometry (Fig. S3, ESI†). The binding constant of current **1**/Cu^{2+} complex is obviously larger than that of DGT/Cu^{2+} (1.58×10^5 M^{-1}), revealing a large improvement of the binding affinity between probe and Cu^{2+}, and therefore made it possible to discriminate the different kinds of amino acids.

Furthermore, to provide more evidence about the improved binding affinity of **1**/Cu^{2+} as well as the binding mode differences between DGT and **1** with Cu^{2+}, density functional theory calculations were performed and reasonable binding configurations were achieved for the complex. It demonstrated that in **1**/Cu^{2+}, the nitrogen in the pyridine ring did participate in the complex formation (Fig. 2). Differently from DGT/Cu^{2+} complex in which DGT form a loop structure with Cu^{2+} by sulfamide, amide and carboxylate group, **1** form loop sturcture with Cu^{2+} through sulfamide, amide and pyridine. The binding mode is very smilar to that of ATCUN/Cu^{2+}.[8] The calculation results reveal that mimicing the ATCUN structure by substituting Trp with pyridine turns out to be successful.

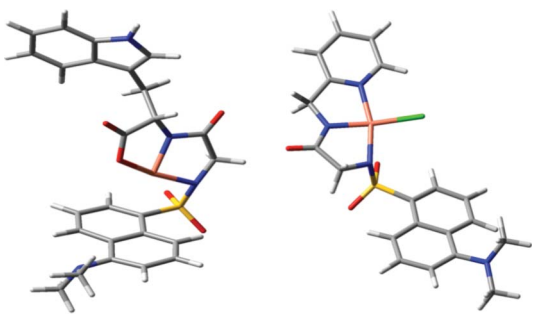

Fig. 2 The simulated structural configuration of **DGT/Cu**$^{2+}$ (*left*) and **1**/Cu^{2+} (*rigth*) complexes.

Then we tried to check the fluorescence responses of **1**/Cu^{2+} to different kinds of amino acids. As shown in Fig. 3, the fluorescence emission of **1** could be released strongly toward the addition of histidine or cysteine, but no such extensive response to other amino acids. Although the addition of cysteine can also recover the fluorescence of **1**, we ascribed the reason to the reduction of the thiol group in cysteine, which reduced the paramagnetic Cu^{2+} to diamagenetic Cu^+ and therefore the fluorescence of **1** quenched by Cu^{2+} was recovered.[11] In addition, the presence of newly produced Cu^+ was further tested by a Cu^+ indicator, neocuproine, in forming an absorption band at around 460 nm for the $[Cu(neocuproine)_2]^+$ complex[12] (Fig. S4, ESI†). Therefore, the results here demonstrated that **1**/Cu^{2+} was sensitive to the reductant as cysteine, which lead it incompatible under reduction conditions as a sensor.

For the fluorescence recovery induced by histidine, we proposed that the stronger binding affinity bewteen **1** and Cu^{2+} ($K = 3.98 \times 10^6$ M^{-1}) exceeded the binding between Cu^{2+} and most of amino acids, which resulted in only histidine could snatch Cu^{2+} from the **1**/Cu^{2+} complex[13] while the other amino acids were faint. Such an obvious fluorescence response of **1**/Cu^{2+} to histidine illustrated the high selectivity of it to different amino acids. When histidine was titrated into the solution containing **1**/Cu^{2+} complex, the fluorescence of **1** restored gradually and which could be fully recovered when the concentration of histidine was added up to 40 μM (Fig. 4). By contrast, the addition of histidine to the solution containing **1**/Hg^{2+} complex could not recover the fluorescence of **1**, indicating that histidine could not extract Hg^{2+} from the complex (Fig.S5, ESI†).

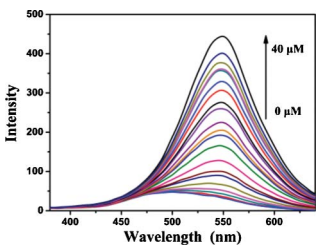

Fig. 4 The fluorescence intensity changes of **1**/Cu^{2+} (20 : 20 μM) upon the addition of histidine in buffer solution, indicating that the fluorescence intensity of **1** can be restored after the coordinated Cu^{2+} were extracted by His.

The new luminescence **1**/Cu^{2+} complex exhibited increase in the intensity of fluorescence at 550 nm and featured its selectivity to Cys and His over other amino acids, which could be further used to distinguish histidine-rich biomolecules, such as His-tagged proteins. To minimize the fluorescence response that originate from the isoelectric point (pI), the protein structure and the presence of certain side chains, GST and that tagged with six His residues at N- terminal were both used to assay the fluorescence response firstly. The presence of 10 μM His-tagged GST could be detected in solution on the basis of a large extent fluorescence recovery (Fig. 5). On the contrary, even at a higher concentration

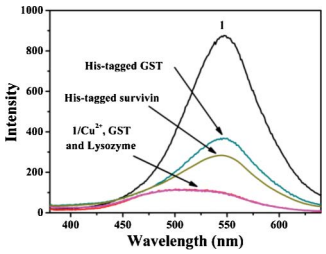

Fig. 5 The fluorescence intensity changes of **1**/Cu^{2+} (20: 20 μM) upon the addition of various protein in buffer solution, indicating that the fluorescence intensity of **1** can be released after the coordinated Cu^{2+} were extracted by the His-tag protein.

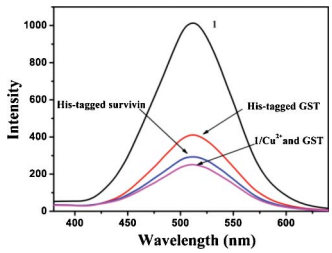

Fig. 6 The fluorescence intensity changes of **1**/Cu^{2+} (20: 400 μM) upon the addition of various protein in buffer solution containing serum, indicating that the fluorescence intensity of **1** can be released after the coordinated Cu^{2+} were extracted by the His-tag protein.

(20 μM) the non-His-tag GST showed no fluorescence recovery. Simultaneously, the His-tagged survivin[14] was also used to clarify the switch-on fluorescence response of **1**/Cu^{2+} to the histidine-rich protein, which show very similar result as the His-tagged GST (Fig. 5).

In addition, in wondering if proteins which are consist of different number and various sequences of amino acids will affect the fluorescence recovery, non-His-tagged BSA, lysozyme and survivin were used as control, respectively. When BSA was added into the solution containing **1**/Cu^{2+} complex, a strong band at 485 nm (data not shown), being far from 550 nm, appeared because of the inclusion of dansyl moiety in the hydrophobic cavity of BSA.[15] However, in the presence of 20 μM non-His-tagged survivin or lysozyme, the fluorescence intensity at 550 nm did not recover. Therefore, we could draw conclusions that the general number and/or sequences of the amino acid in protein, except BSA, had no obvious effect on the fluorescence recovery of **1**/Cu^{2+}. The **1**/Cu^{2+} complex had specific fluorescence response to the His-tagged proteins in buffer solutions.

Finally, in order to demonstrate that **1**/Cu^{2+} has a potential application in protein detection practically, the complex was applied to the solution containing serum to mimic the conditions of real case. When **1** was added into buffer solution containing standard foetal bovine serum (v/v = 1 : 1), a strong fluorescence band appeared at 511 nm, other than 550 nm, because of the inclusion of dansyl moiety in BSA. Similarly as the response in buffer solution, further addition of Cu^{2+} quenched the fluorescence; however, identical quenching level can be achieved only after the concentration of Cu^{2+} was increased up to 400 μM, which may relate to the competitive binding between **1** and BSA. Once we increase the ratio of serum in buffer solution, more Cu^{2+} will need. In such a case, when the his-tagged survivin and GST was added into the solution, respectively, the fluorescence of **1** could be recovered partly either. But the non-his-tagged GST had no obvious effect on the fluorescence recovery (Fig. 6). The results indicated that **1**/Cu^{2+} could be used to detect his-tagged protein in the real system although the sensitivity needs to be improved further.

In summary, we have developed a new luminescent switch-on **1**/Cu^{2+} complex, which could be used to distinguishing histidine and cysteine from other amino acids, and further to detecting proteins with and without His-tags either in buffer or foetal bovine serum solution, indicating that **1**/Cu^{2+} had potential application in real sample.

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