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New fluorescent probe based on rhodamine derivative for detection of both Cu^{2+} and L-Methionine and living cells imaging

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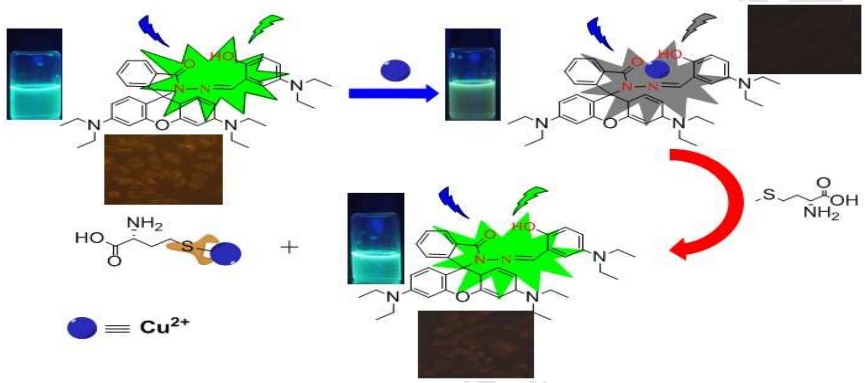
A new fluorescent rhodamine-based derivatives (RAD) probe for highly sensitive and selective detection of Cu^{2+} and L-Methionine (L-Met) was developed with the detection limits can reach as low as 12 nM for Cu^{2+} and 5 nM for L-Met, In addition, the RAD probe can be further applied to cell imaging owing to its photostability and low cytotoxicity.



New fluorescent probe based on rhodamine derivative for detection of both Cu^{2+} and L-Methionine and living cells imaging

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Abstract: This paper developed a new fluorescent rhodamine-based derivatives (RAD) probe for highly sensitive and selective detection of Cu^{2+} and L-Methionine (L-Met). The detection limits can reach as low as 12 nM for Cu^{2+} and 5 nM for L-Met, In addition, the RAD probe can be further applied to cell imaging owing to its photostability and low cytotoxicity.

Keywords: Rhodamine-based derivatives; Sensitive; Selective; Detection limits; Cell imaging

1. Introduction

The content of copper ion is only lower than Zn^{2+} and Fe^{2+} in living organisms[1] and plays an important role in biological processes[2]. However, excessive Cu^{2+} can also result in serious damage on living organisms, such as respiratory ailments[3], kidney failure[4], Wilson's disease[5], Alzheimer's disease[6], and Menke's disease[7]. In addition, the study of amino acids have drawn much attention in recent years due to their crucial roles and functions in a wide range of biological processes[8-9]. It is well known that a small-molecular-weight biological thiol-containing amino acid, L-Methionine (L-Met) is essential in maintaining biological redox homeostasis, an abnormal level of L-Met can lead to many diseases including slow growth, liverdamage, skinlesions, and so on[10-12]. Therefore, accurate and rapid detection of trace Cu^{2+}

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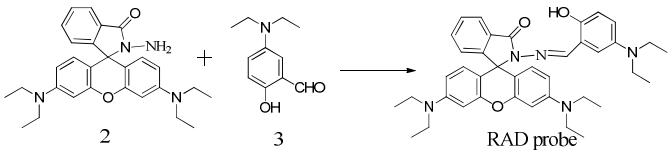
and L-Met are very important for disease surveillance in living organisms. So far there have been many reports about the detection of copper ions[13], as for L-Met is rare. Therefore, a simple and direct method for in situ detecting both Cu^{2+} and L-Met is of great importance.

Until now, many sophisticated analytical techniques have been widely used for detecting metal ions and biomolecules such as atomic absorption spectrometry[14], inductively coupled plasma emission spectrometry[15], neutron activationanalysis[16], anodic stripping voltammetry[17]. But some drawbacks limit the application range of these methods due to expensive instruments, time-consuming, well-controlled experimental conditions. Compared with these approaches, the fluorescent analysis methods have attracted great interests in its several outstanding advantages on low cost, simplicity, fast real time, high sensitivity and selectivity[18]. Therefore, fluorescence analysis method has been regarded as one of the most effective detection techniques for detecting trace heavy metal ions and amino acids.

As the traditional fluorescent dye, rhodamine-based derivatives have attracted considerable attention due to their good photo-physical properties including high fluorescence quantum yield, visible absorption and fluorescence emission[19]. As is known to all, the rhodamine framework is easily modified at a well-defined molecular level for constructing “off-on” fluorescent or colorimetric chemosensors due to its special structural feature[20]. Up to now, a number of rhodamine spirolactam-based probes have been designed and effectively applied for various metal cations detction[21]. In this paper, a novel rhodamine derivative (RAD, compound 1) with strong solid fluorescence emission was designed and synthesized. The obtained RAD can exhibit direct and visual detection of trace Cu^{2+} with high selectivity and sensitivity, and the resulting *in situ* RAD-Cu^{2+} complex could further show high sensitive response behavior on L-Met. The synthesis

process of RAD was illustrated in Scheme 1.



Scheme 1. Synthesis and the structure of RAD probe

2. Materials and methods

2.1. Reagents and chemicals

4-(Diethylamino)-salicylaldehyde, rhodamine B, and 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny ltetrazolium bromide (MTT) were purchased from Aladdin Chemistry Co. Ltd (Shanghai, China) and were used without further purification. Other inorganic salts, amino acids were obtained from commercial sources. Double-distilled water was used in all of the experiments. All samples were prepared at room temperature and were shaken for 1 min before the test.

2.2. Apparatus

^{1}H NMR spectra were recorded on a Bruker AN-400 MHz instrument for solutions in d_6-Dimethyl Sulfoxide (DMSO), using tetramethylsilane as an internal reference. Electron impact mass spectra were conducted on MAT-212 spectrometer. Elemental analyses were done at Vario EL Ⅲ. Ultraviolet-Visible (UV-vis) absorption spectra were measured with a Varian Cary 50 spectrophotometer at 1 cm of the light path length. Fluorescence spectra were recorded on Varian Cary Eclipse fluorescence spectrophotometer with an excitation wavelength of 400 nm.

2.3. Synthesis of RAD

Rhodamine B hydrazide was synthesized according to a previous described procedure[22].The synthesis of **RAD** is as follows: Compound 2 (0.46 g, 1 mmol), 4-(Diethylamino)-salicylaldehyde (0.19 g, 1 mmol) were added into 30 mL ethanol and refluxed

for 24 h. After cooled to room temperature, the mixture was filtered and washed with ethanol. The crude product was recrystallized from DMF and dried in vacuum to give an orange solid 0.55 g. Yield: 84%. ^{1}H NMR (400Hz, d_6-DMSO), δ: 1.02-1.12 (m, 18H, -CH_3-), 2.85-3.27 (m, 12H, -CH_2-), 6.08-6.43 (m, 8H, Ar-H), 7.02-7.21 (m, 5H, Ar-H), 8.39 (s, 1H, -N=CH), 11.12 (s, 1H, -OH). ^{13}C NMR (75 MHz, $CDCl_3$), 11.6, 11.9, 12.2, 12.3, 12.5, 12.6, 46.8, 47.1, 47.3, 47.5, 47.7, 47.8, 76.3, 97.7, 97.9, 106.5, 106.7, 112.9, 114.3, 114.6, 118.0, 118.3, 119.4, 128.0, 128.3, 128.7 ,128.8, 129.2, 131.3, 132.6, 139.5, 142.3, 143.3, 149.4, 149.6, 150.6, 151.6, 151.9, 170.4; MS (ESI, m/z): 632.5 (M^++1); Elemental analysis. Found: C, 74.17; H, 7.21; N, 11.13.

2.4. UV-vis and fluorescent experiments

RAD was dissolved in DMSO at an ambient temperature to obtain a stock solution (20.0 μM). The stock solution of metal ions was prepared in a phosphate buffer (pH=6.8), and the concentration of was 20.0 μM. Test solutions were prepared by placing 3 mL of the stock solution into a cuvette. All experiments were performed at room temperature.

2.5. Cells Assay

HeLa cells were harvested (the cell density was adjusted to 10^5 cells per mL) and seeded in a 96-well plate (90 μL $well^{-1}$) overnight and RAD suspensions with different concentrations (20, 40, 60, 80, and 100 μg mL^{-1}) were then added. The cells were cultivated for 24 h, and 20 μL of 1 mg/mL MTT solution was then added to each cell well. After the cells were incubated for 4 h, the culture medium was discarded, and 150 μL of dimethylsulfoxide was added. The resulting mixture was shaken for 15 min in the dark at room temperature, and its optical density (OD) was measured by using a microplate reader (Thermo).

Human Hela cells were cultured in DMEM containing 10% fetal bovine serum in a

humidified incubator at 37 °C and 5% CO_2. The RAD suspension was injected into the well of a chamber slide with the final RAD concentration of 20 μg mL^{-1}. After incubation for 24 h, the cells were washed 3 times using phosphate buffered saline. The fluorescence images were acquired using an excitation wavelength of 488 nm.

3. Results and discussion

3.1. UV-vis and fluorescence properties

The coordination formation of RAD ligand with Cu^{2+} was first investigated by UV-vis absorption spectroscopy in DMSO solution. As shown in Fig. 1, we found that the absorption bands of RAD located in 275 and 380 nm gradually decreased with a steadily increasing new band centered at 437 nm upon the addition of Cu^{2+} into the solution of RAD. Meanwhile, the colour of RAD-Cu^{2+} complex solution was changed from kelly to brown (Fig.1 inset), which could be clearly observed by the naked eye for direct and visual recognition of Cu^{2+}.

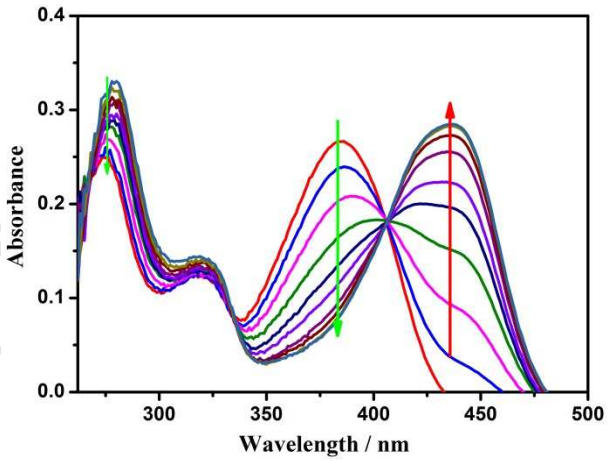


Fig. 1 UV-vis titration spectra of RAD (10 μM) upon the gradual addition of Cu^{2+} (0-40 nM).

Fluorescence titration experiments were first performed to investigate the sensitivity of RAD probe towards Cu^{2+}. As shown in Fig.2a, the as-prepared RAD initially exhibited a characteristic fluorescence emission band at 506 nm under the excitation at 400 nm. Upon the addition of various concentrations of Cu^{2+} to RAD solution, obvious “turn-off” fluorescence quenching

response can be observed. The relationship of fluorescence intensities in the absence and presence of Cu^{2+} could be depicted by Stern–Volmer equation: $F_0/F_1=1+K_q[Q]$, where F_0 and F_1 are the fluorescence intensities of RAD in the absence and presence of Cu^{2+}, respectively; [Q] is the concentration of Cu^{2+}, and K_q is the Stern-Volmer constant. The calibration curve in Fig.2b shows a linear relationship (R^2=0.986) of F/F_0 versus the concentration of Cu^{2+} over the range from 0 to 40 nM. The detection limit (3σ) for Cu^{2+} was calculated to be as low as 12 nM. The results demonstrate that RAD can serve as a fluorescent probe for detecting Cu^{2+} with quick response and high sensitivity. In order to test the practical utility of Cu^{2+} recognition by RAD probe, a set of comparable experiments were further carried out by using other relevant metal ions including Ag^{+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Pb^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}. The results demonstrated that all these metal ions caused only little fluorescence emission variations (Fig. S1), which indicated that the RAD probe can be effectively used to detect trace Cu^{2+} ions.

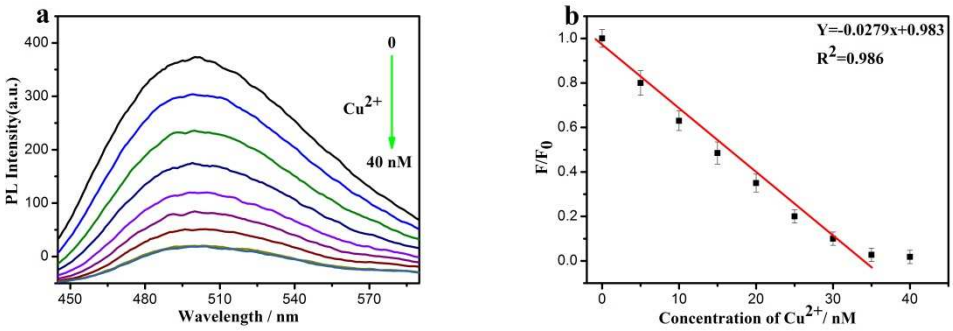


Fig.2 (a) Fluorescence response of RAD in the presence of increasing concentration of Cu^{2+} (from up to down the concentration of Cu^{2+} is 0, 5, 10, 15, 20, 25, 30, 35 and 40 nM). (b) The linear relationship of F/F_0 versus the concentration of Cu^{2+} over the range from 0 to 40 nM. λ_{ex}=400 nm.

As is evident from Fig. 3a, it is clearly found that L-Met can exhibit the “turn-on” fluorescence response behavior on the recovery of the *in situ* RAD-Cu^{2+} system. The fluorescence intensity of the system appeared gradual enhancement with the increase of L-Met concentration. The relationship between the fluorescence intensity changes of *in situ* RAD-Cu^{2+} system and the

concentration of L-Met was shown in Fig. 3b. The enhancement of fluorescence emission is proportional to the concentration of L-Met in the range from 0 to 40 nM (R^2=0.984), and the detection limit is 5 nM. To further evaluate the selectivity of L-Met, herein the response behaviors of other chosen amino acids (threonine，lysine，glutamate，tryptophan) and glutathion on the *in situ* RAD-Cu^{2+} system were also investigated. It can be clearly observed that only thiol-containing species can result in the "turn-on" fluorescence enhancement response (Fig. S2). Most importantly, when adding other amino acids or thiol-containing species, the fluorescence signal of the *in situ* RAD-Cu^{2+} system was lower than that of the system in the presence of L-Met, which also demonstrated that the designed *in situ* RAD-Cu^{2+} sensing system can exhibit excellent selectivity only towards L-Met.

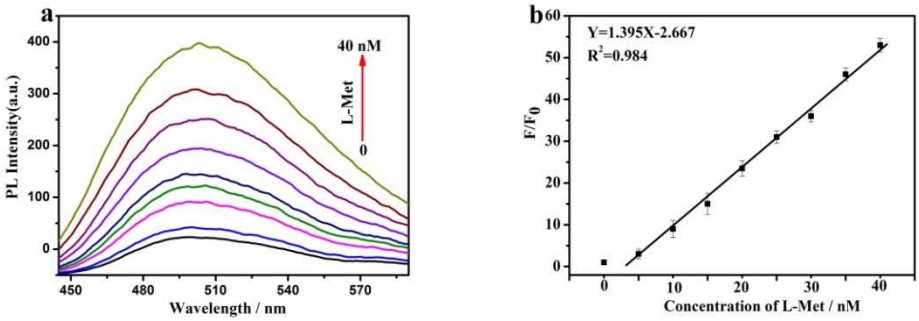
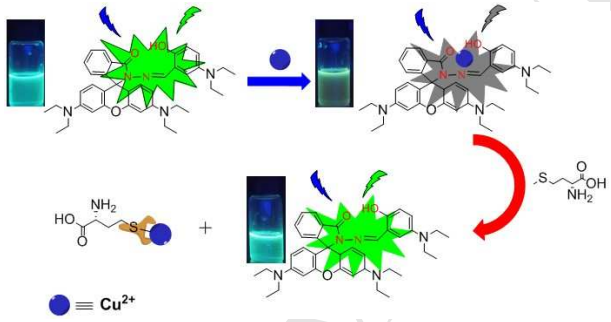


Fig.3 (a) Fluorescence response of RAD-Cu^{2+} system in the presence of increasing concentration of L-Met (from up to down the concentration of L-Met is 0, 5, 10, 15, 20, 25, 30, 35 and 40 nM). (b) The linear relationship of F/F_0 versus the concentration of L-Met over the range from 0 to 40 nM(λ_{ex}=400 nm).

In this paper, we further investigated the proposed fluorescence response mechanism of RAD probe on the detection of Cu^{2+} and L-Met. As was reported[23], hydroxyl, carboxyl and imino group of compounds can be influenced by metal ions through coordination reaction and further influence the fluorescence emission intensity of the original compound. Herein, as illustrated in Scheme 2, when RAD is free, the aqueous solution can show strong fluorescence emission at 506 nm with the excitation wavelength about 400 nm. With the addition of Cu^{2+}, the hydroxyl and

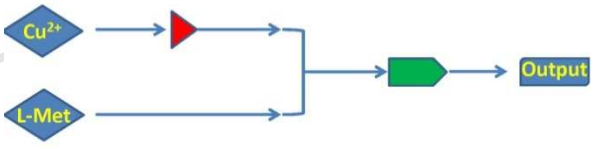
amidogen of RAD can coordinate with Cu^{2+} easily and cause strong fluorescence quenching. As we know, thiol group could exhibit strong binding preference towards Cu^{2+} by forming Cu^{2+}-S bond[24]. We further add L-Met to the RAD-Cu^{2+} system, the competitive interaction between Cu^{2+}, L-Met, and RAD disturbs the interaction between Cu^{2+} and RAD, Cu^{2+} was then able to be removed from the RAD, and thus the fluorescence probe of the system could be recovered. The different fluorescent responses upon the addition of Cu^{2+} and L-Met make RAD possible to be used for Cu^{2+} and L-Met detection.



Scheme 2 Mechanism of the "off–on" detection of Cu^{2+} and L-Met.

3.2. Logic gate

Based on the different fluorescence responses of RAD sensor towards Cu^{2+} and L-Met, a type of logic gate that incorporating a NOT gate and an OR gate was designed. Low fluorescence intensity was observed only in the presence of 1.0 equiv. of Cu^{2+} leading to the output as "0". When L-Met was introduced to the system, fluorescence is restored, resulting in output "1" [25].



Input		output	
Cu^{2+}	L-Met	RAD fluorescence	
0	0	High(19.8)	1
1	0	Low(1.2)	0
0	1	High(20)	1
1	1	High(19)	1

Fig.4 Logic gate with Cu^{2+} (40 nM) and L-Met (40 nM) as "input".

3.3. Biocompatibility and Application of RAD in Cell Imaging

Herein, the RAD probe with strong fluorescence emission was further applied for living cell bioimaging. MTT assay was first carried out to evaluate the cytotoxicity of the RAD to Hela cells. As is illustrated in Fig. S3, the viability of Hela cells declined by only < 8% even when the concentration of RAD was increased up to 100 μg mL^{-1}, indicating that the concentration of RAD in vitro is much higher than that required for the imaging of living cells. And then the potential application in living cells was researched. Evaluation of the bioimaging applicability of RAD was performed by using a Nikon eclipse inverted fluorescence microscope. As shown in Fig. 5, strong fluorescence in the cells was observed after the cells were incubated with RAD. In contrast, weak fluorescence was observed when Hela cells were further incubated with Cu^{2+} (10 nM) for another 8 h. However, the fluorescence of cells could be quickly recovered after incubation with 5 nM L-Met for 20 min at 37 °C. Therefore, these results revealed that RAD has the potential for the detection of copper ion in living cells and their complex RAD-Cu^{2+} would also be an excellent monitoring system to visualize L-Met in living cells.

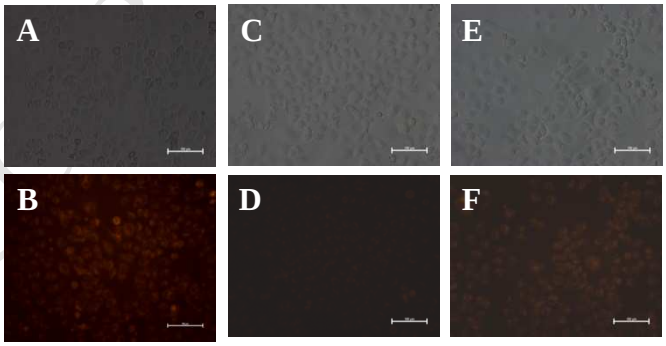


Fig .5 Fluorescence microscope images of HeLa cell with different treatment. (a and b) Bright-field image and fluorescence mode of HeLa cells treated with RAD (20 μM). (c and d) Bright-field image and fluorescence mode of HeLa cells treated with RAD (20 μM) and Cu^{2+} (5 nM). (e and f) Bright-field image and fluorescence mode of HeLa cells treated with RAD (20 μM) , Cu^{2+} (5 nM) and L-Met (5 nM). λ_{ex}=510 nm. Scale bar: 100 μm.

4. Conclusions

Copper ion and L-Met play important roles in the process of life, trace changes of Cu^{2+} or

L-Met could cause many diseases. So far, no fluorescent probe can detect both Cu^{2+} and L-Met simultaneously. In this paper, RAD was synthesized and used for "off–on" probe detection of Cu^{2+} and L-Met with high sensitivity and selectivity. Cu^{2+} can quench the fluorescence of RAD through coordination interaction, while L-Met can show stronger binding ability to Cu^{2+} and recover the fluorescence of RAD. The probe further could be applied to detect Cu^{2+} and L-Met in Hela cells *via* intracellular fluorescent imaging. The strategy presented is facile, rapid, low cost, and environmentally friendly, therefore it can be employed to detect Cu^{2+} and L-Met in real samples.

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