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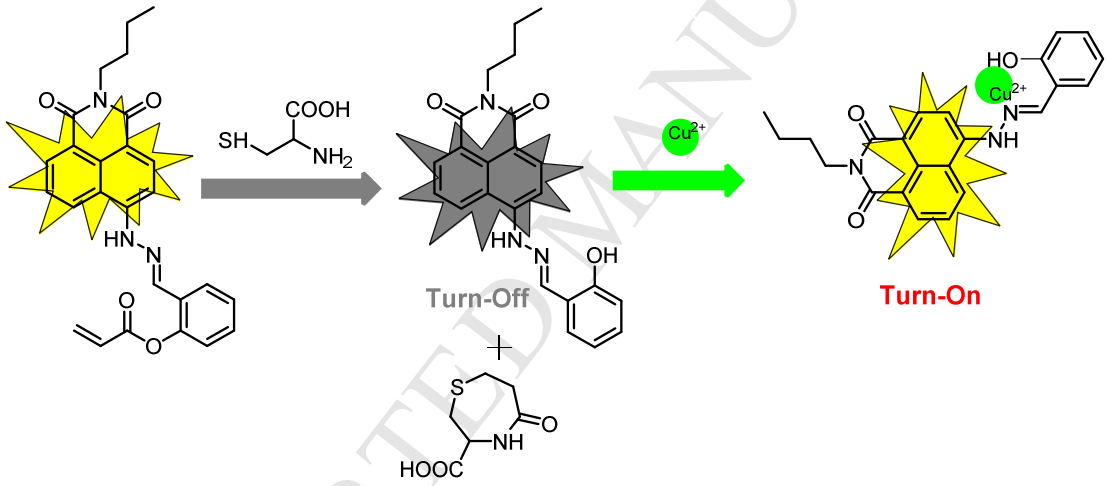
1,8-naphthalimide derivative (NAD) was developed for highly sensitive and selective detection of Cys and Cu^{2+} with the detection limits as 25 nM for Cys and 11 nM Cu^{2+}. In addition, the NAD probe can be further applied to cell imaging owing to its photostability and low cytotoxicity.



A sensitive fluorescent probe for cysteine and Cu^{2+} based on 1,8-naphthalimide derivatives and its application in living cells imaging

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Abstract: A new 1,8-naphthalimide derivative (NAD) was synthesized and employed as a highly sensitive and selective probe for the detection of cysteine (Cys) and Cu^{2+}. The terminal olefin of NAD could react with Cys and resulting in fluorescence quenching, with the adding of Cu^{2+}, the schiff base unit of NAD could coordinate with Cu^{2+} and lead to a highly selective fluorescence "turn-on" response. The detection limits reached as low as 25 nM for Cys and 11 nM for Cu^{2+}. In addition, the NAD probe can be further applied to cells imaging owing to its photostability and low cytotoxicity.

Keywords: 1,8-naphthalimide-based derivatives; Fluorescent probe; Detection limits; Cells imaging

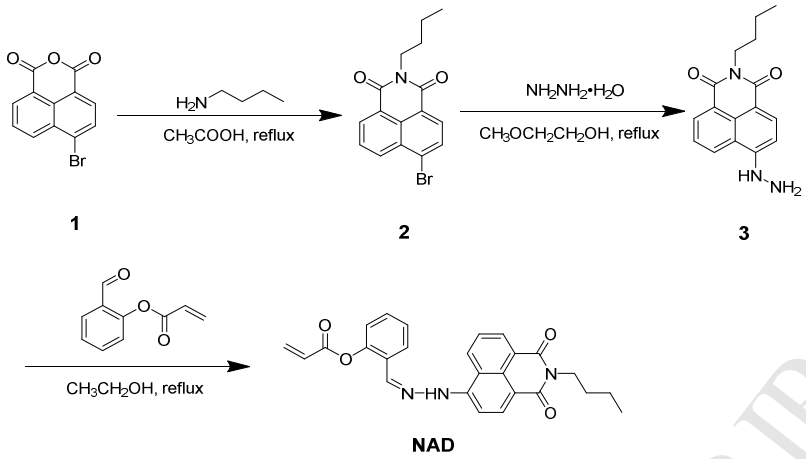
1. Introduction

Among the essential heavy metal ions in the human body, copper ion plays an important role in biological process[1-3]. Copper deficiency may lead to hematological manifestations and a wide variety of neurological problems, while excessive Cu^{2+} can also be potentially toxic to living cells and resulting in oxidative stress cardiovascular disorders and neurodegenerative diseases including Alzheimer'sdisease[4], Menke's disease[5]and prion diseases[6]. In recent years, amino acids have drawn much attention due to their crucial roles and play the crucial roles in various physiological processes [7-11]. In these amino acids, Cys as an essential amino acid could be used in protein synthesis[12], detoxification[13], metal binding[14-15], post-translational modification

and metabolism widely[16-17]. Abnormal level of Cys can lead to many diseases including slow growth, liver damage, skin lesions, and so on[18-20]. Therefore, accurate and rapid detection of trace Cu^{2+} and Cys are very important for disease surveillance in living organisms.

Until now, many analytical methods including atomic absorption spectrometry [21], inductively coupled plasma emission spectrometry[22], neutron activation analysis[23], and fluorescence method [24-31] have been established to detect the Cu^{2+} and Cys. Compared with other technologies, fluorescent analysis methods have attracted great interests as to its advantage of simplicity, high sensitivity and low lost. Therefore, looking for ideal fluorescent probe becomes a hot research topic.

As traditional fluorescent dye, 1,8-naphthalimide-based derivatives have attracted considerable attentions due to their desirable optical properties such as high photostability, large Stokes' shift, high fluorescence quantum yield, visible absorption and fluorescence emission[32-35]. In this paper, a novel 1,8-naphthalimide derivative (NAD) with strong fluorescence emission was designed and synthesized. The obtained NAD exhibit high selectivity and sensitivity towards direct and visual detection of trace cysteine, and the resulting *in situ* new NAD derivative was found to further show highly sensitive response behavior on Cu^{2+}. The synthetic route of NAD was illustrated as shown in Scheme 1.



Scheme 1 Synthesis and structure of NAD

2. Materials and methods

2.1. Reagents and chemicals

NAD, Butylamine, Hydrazine hydrate and 2-methoxyethanol were purchased from Aladdin Chemistry Co. Ltd (Shanghai, China) and were used without further purification. Double-distilled water was used in all of the experiments. Other inorganic salts, amino acids were obtained from Sinopharm. All samples were prepared at room temperature and were shaken for 1 min before the test.

2.2. Apparatus

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer instrument for solutions in Dimethyl Sulfoxide (DMSO), using tetramethylsilane as an internal reference. Electron impact mass spectra were run on MAT-212 spectrometer. 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 456 nm. Elemental analysis was recorded on VARIO EL III. High-resolution mass spectra (HRMS) were obtained on a MALDI-TOF/TOF Ultrafle Xtreme (Bruker USA).

2.3. Synthesis of NAD

Compound **3** and 2-formylphenyl acrylate were synthesized according to a previous described procedure [36]. The synthetic route of NAD was outlined in Scheme 1, and the detailed procedures are as follows: a mixture of compound **3**(0.56 g, 2.0 mmol), 2-formylphenyl (0.39 g, 2.2 mmol) and 0.5 mL acetic acid was added into 30 mL ethanol then refluxed for 36 h. After cooled to temperature, the mixture was filtered and washed with ethanol. The crude product was recrystallized from dimethylformamide and dried in vacuum to give an orange solid (**NAD)**. Yield: 72%. Mp:315-317℃. ^{1}H NMR (400 MHz, d_6-DMSO), δ: 0.91 (t, 3H, J = 4Hz, CH_3), 1.29-1.38 (m, 2H, CH_2), 1.55-1.63 (m, 2H, CH_2), 4.01 (t, 2H, J = 6Hz, CH_2), 6.28-6.31 (m, 1H, CH), 6.54-6.60 (m, 1H, CH), 6.65-6.70 (m, 1H, CH), 7.27 (t, 1H, J = 4Hz, ArH), 7.41 (t, 1H, J = 6Hz, ArH), 7.46-7.51 (m, 1H, ArH), 7.69 (d, 1H, J = 8Hz, ArH), 7.76 (t, 1H, J = 8H, ArH), 8.07-8.10 (m, 1H, ArH), 8.33 (d, 1H, J = 8Hz, ArH), 8.45 (d, 2H, J = 8Hz, ArH), 8.72 (d, 1H, J = 8Hz, N=CH), 11.50 (s, 1H, NH); ^{13}C NMR (100 MHz, d_6-DMSO),164.6, 163.9, 163.2, 148.8, 146.5, 138.5, 134.8, 133.6, 131.2, 130.8, 129.4, 128.6, 127.9 127.5, 127.0, 126.9, 125.3, 123.7, 122.4, 119.0, 111.8, 107.5, 30.2, 20.3, 19.0, 14.2; MS (ESI, m/z): 442.2 (M^++1). Elemental analysis (%) calcd for $C_{26}H_{23}N_3O_4$: C 70.74, H 5.25, N 9.52; found: C 70.69, H 5.29, N 9.50. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{26}H_{23}N_3O_4$: 442.1768; found: 442.1783.

2.4. UV-vis and fluorescent experiments

The stock solution of 10 μM probe NAD was prepared in DMSO/H_2O (9:1, v/v). Stock solutions (10.0 μM) of metal ions and amino acids were prepared in distilled water. 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

Human Hela cells were cultured in dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum in a humidified incubator at 37 °C and 5% CO_2. Cells were plated in 96-well plates and incubated with the NAD concentration of 10 μM for 24 h at 37 °C, washed with PBS 3 times. The fluorescence images were acquired by using a Nikon eclipse inverted fluorescence microscope.

3. Results and discussion

3.1 Fluorescent sensing of Cys

The availabilities of NAD as a highly selective probe for amino acids were researched by using fluorescence spectra. The fluorescence spectra response of NAD with the concentration of 10 μM to various amino acids (Cys, Met, Thr, Lys, Glu, Try, His , Phe, Gly, Leu, Val, Ben, Ala, Leu) (10 μM) were measured respectively. It could be found that only Cys could induce obvious fluorescence weakening while other amino acids showed slight changes which indicated this probe can effectively detect Cys. To examine the sensing behavior of the probe to Cys, fluorescence titration experiments of the probe (10 μM) with Cys were carried out. The fluorescence spectra with different concentrations of Cys were studied and shown in Fig.1(a). It can be seen clearly that the probe initially exhibited a characteristic fluorescence emission band at 536 nm under the excitation at 456 nm. The fluorescence intensity decreased significantly with increasing the concentration of Cys from 0 to 200 nM. A good linear relationship between the fluorescence intensity at 535 nm and the concentration of Cys in the range of 0-80 nM was obtained (R = 0.9912) The calibration curve was illustrated in Fig.1(b) with the concentration from 0 to 80 nM and the detection limit was calculated to be as low as 25 nM (based on S/N = 3). which

demonstrated that NAD probe can be used for detecting trace Cys with high sensitivity.

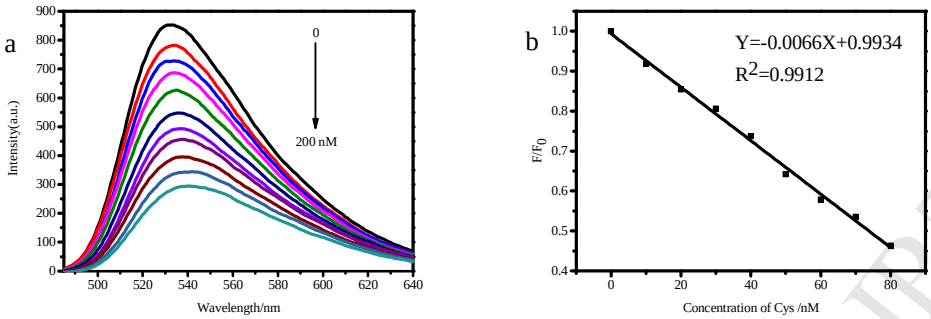


Fig.1. (a) Fluorescence response of probe in the presence of increasing concentration of Cys (from up to down the concentration of Cys is 0, 10, 20, 30, 40, 50, 60, 70, 80, 140 and 200 nM); (b) The linear relationship of F/F_0 versus the concentration of Cys over the range from 0 to 80 nM.

3.2 UV-vis spectra

In this paper, the UV–vis absorption spectral of NAD to Cys was further researched in DMSO/H_2O (9:1, v/v)**.** As shown in Fig. 2, the absorption band of NAD located in 454 nm and gradually increase with red shift to 464 nm when Cys was added (0-200 nM) into the solution of NAD. Meanwhile the colour of the new NAD derivative′ solution changed from yellow to orange, which could be clearly observed by the naked eye for direct and visual recognition of Cys.

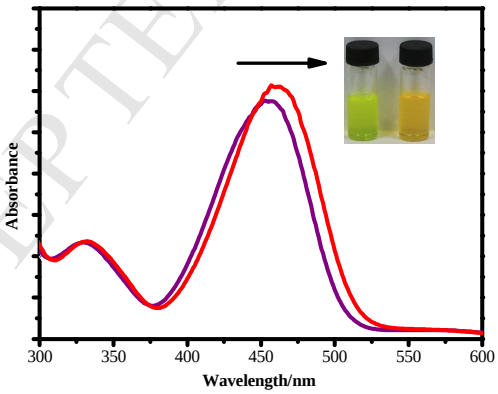


Fig.2. UV-vis spectra of the probe (10 μM) brfore and after adding 200 nM Cys. inset was the photo of NAD before and after adding Cys.

3.3 Fluorescent sensing of Cu^{2+} ion

A large number of reports[37-39] confirmed that copper could react with Schiff base through coordination with the amino of Schiff base. In this paper, NAD-Cys may be applied as a fluorescent probe to detect copper. As showed in Fig.3(a), with the concentration of copper

increases from 0 to 180 nM, the fluorescence of NAD-Cys system recovery gradually and the enhancement of fluorescence emission is proportional to the concentration of Cu^{2+} in the range from 60 to 160 nM (R^2=0.990), with the detection limit is 11 nM (based on S/N = 3). In addition, a set of comparable experiments were further carried out by using other relevant metal ions including Na^+, K^+, Cu^{2+}, Fe^{3+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ag^+, Zn^{2+}, Ni^{2+} and Pb^{2+}. The results showed that only Cu^{2+} could cause a strong fluorescence change (Fig.S3) which proved that this NAD-Cys systems could be used for detection of copper ions with high selectivity.

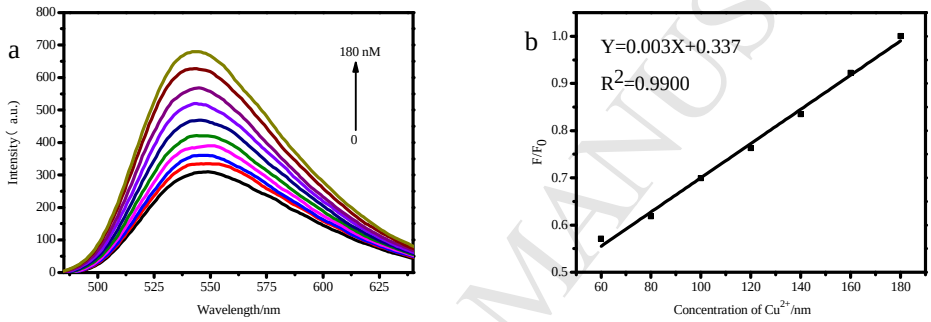
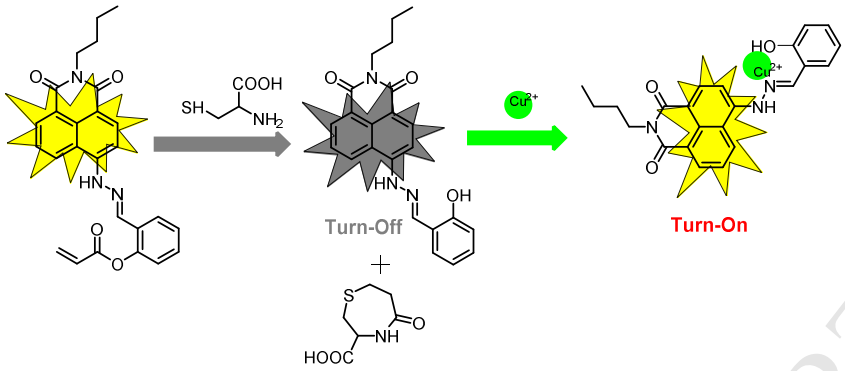


Fig.3. (a) Fluorescence response of the probe in the presence of increasing concentration of Cu^{2+} (from down to up the concentration of Cu^{2+} is 0, 20, 40, 60, 80, 100, 120, 140, 160 and 180 nM); (b) The linear relationship of F/F_0 versus the concentration of Cu^{2+} over the range from 60 to 160 nM.

3.4 Mechanism for the analysis of Cys and Cu^{2+}

In this paper, we further investigated the mechanism for the analysis of Cys and Cu^{2+}. It is well known that the condensation of acrylates with Cys can be used for the preparation of substituted 1,4-thiazepine[40]. As shown in Scheme 2, on the detection of Cys, the conjugate addition of Cys to acrylate to generate thioethers and the ensuing intramolecular cyclization reaction will cause strong fluorescence quenching. And with the addition of Cu^{2+}, the hydroxyl oxygen atom and Shift base nitrogen atoms can coordinate with Cu^{2+} easily, which can induce an increasing fluorescence. The different fluorescent responses make NAD possible to be used for Cys and Cu^{2+} detection.



Scheme 2. Mechanism of "off -on" detection of Cys and Cu^{2+}.

3.5 Cell Imaging

To further demonstrate the practical applicability of the probe in biological systems, fluorescence imaging experiments were carried out in Hela cells. We first investigated the application of NAD for live cell bioimaging. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was first carried out to evaluate the cytotoxicity of NAD to Hela cells. As illustrated in Fig. S4, the viability of Hela cells declines by only <8% even when the concentration of NAD was increased up to 100 μg/mL, indicating that the concentration of NAD *in vitro* is much higher than that required for the imaging of living cells. As shown in Fig.4, Upon incubating the HeLa cells with the probe (10 μM) for 8 h, strong green fluorescence was observed inside the cells. In contrast, weak fluorescence was observed when Hela cells were further incubated with Cys (10 μM) for another 8 h, indicating that the probe could reacted with intracellular Cys. However, the fluorescence of cells could be quickly recovered after incubation with Cu^{2+} (10 μM) for another 20 min. These imaging experiments indicated that this probe could be applied to detecting Cys in cells and its complex NAD-Cys would also have the potential for the detection of Cu^{2+} in living cells.

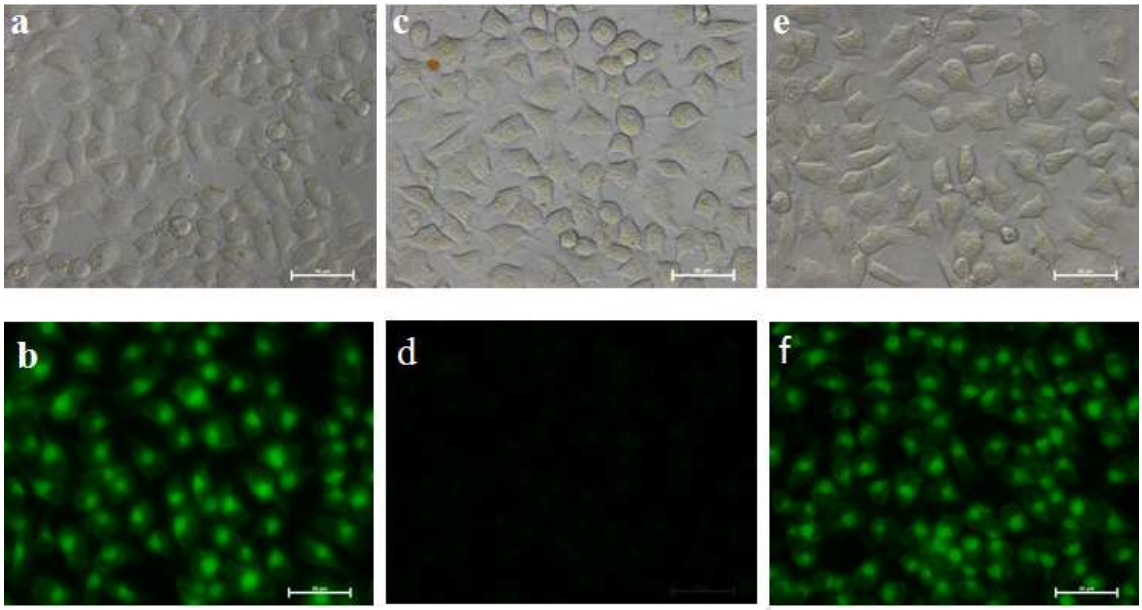


Fig.4. Fluorescence microscope images of HeLa cell with different treatment. (a and b) Bright-field image and fluorescence mode of HeLa cells treated with NAD (10 μM). (c and d) Bright-field image and fluorescence mode of HeLa cells treated with NAD (10 μM) and Cys (10 μM). (e and f) Bright-field image and fluorescence mode of HeLa cells treated with NAD (10 μM)、Cys (10 μM) and Cu^{2+}(10 μM). Ex=488 nm. Scale bar: 50 μm.

4. Conclusions

In conclusion, a new 1,8-naphthalimide-based fluorescent probe was synthesized and be used for detection of cysteine with high sensitivity and selectivity. Cysteine can quench the fluorescence of the probe by reacting with terminal olefin of NAD, The formed NAD-Cys show stronger binding ability to Cu^{2+} and recover the fluorescence of NAD. The NAD further could be developed and applied to detect Cys and Cu^{2+} in Hela cells via intracellular fluorescent imaging successfully.

Supporting Information.

Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at http:// dx.doi.org/10.1016/j.tet.2016.xx.xx

Acknowledgments

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