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Full Length Article

A new ensemble approach based chemosensor for the reversible detection of bio-thiols and its application in live cell imaging

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ABSTRACT

Based on an aldazine-copper chemosensing ensemble (**NP**-Cu^{2+}), a new fluorescence chemosensor for the detection of biothiols (Cys, Hcy and GSH) was designed and synthesized. In aqueous solution, the ligand **NP** exhibited high selectivity toward Cu^{2+} ions by forming a 2:1 complex, accompanied with a dramatic fluorescence quenching and a notable bathochromic-shift of the absorbance band. Due to the high affinity of thiols and copper, the specific interaction of thiols (Cys, Hcy and GSH) with **NP**-Cu^{2+} ensemble led to the liberation of the **NP**. As the result, recovery of fluorescence and UV–vis absorbance was observed. The detection limits of **NP**-Cu^{2+} to Cys, Hcy and GSH were estimated to be 1.5 μM, 1.8 μM and 2.2 μM, respectively. The fluorescence "OFF–ON" circle can be repeated to a minimum of 5 times by the alternative addition of thiols and Cu^{2+}, implying that **NP**-Cu^{2+} is a recyclable chemosensor for thiols. Results of fluorescence microscopy imaging suggested that **NP**-Cu^{2+} has potential to be used as a powerful tool for the detection of intracellular thiols.

1. Introduction

As the building block for the proteins, biological thiols, including cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) play crucial roles in physiological systems, such as biological redox homeostasis, biocatalysis, metal binding and post translational modifications [1–3]. It has been reported that the abnormal levels of biothiols are implicated with various diseases [4]. The deficiency of biothiols is involved in slow growth in children, hair depigmentation, edema, lethargy, liver damage, loss of muscle and fat, skin lesions, and weakness [5–7]. While the elevated levels of intracellular biothiols are causal to or can exacerbate pathogenesis of Alzheimer's disease, cardiovascular disease, osteoporosis, and neurotoxicity [8–14]. Therefore, the development of simple and effective methods for the rapid and sensitive detection of biothiols in aqueous solution, and especially in living systems are of great importance for understanding their functions in cells and disease diagnosis [15].

Fluorescent analysis by using responsive chemosensor has been recognized as one of the most promising technologies for the study of specific biological molecules in live organisms. In the last two decades, enormous efforts have been devoted to the development of fluorescent chemosensors for the detection of thiols based on various chemical mechanisms, such as Michael addition [16–18], cyclization with aldehyde [19–21], disulfide bond cleavage reaction [22,23], binding with nanoparticles [24,25], cleavage of sulfonamide and sulfonate ester [26–30]. Recently, chemosensing ensemble-based displacement approach has been reported as one of new mechanisms for the thiols detection [31–40]. For the displacement strategy, the receptor (metal cation) is noncovalently attached to the fluorescent indicator by forming a so-called "chemosensing ensemble", which is non-fluorescent due to metal cation-induced fluorescence quenching [41,42]. In the presence of thiols, the detachment of metal ions led to the liberation of fluorophore, and thus the fluorescence was recovered. Based on this strategy, we have developed a series of chemosensors for the detection of anions in live systems [43–45]. In this work, we shifted our interest to the development of fluorescent chemosensor for the identification of biothiols from other amino acids in

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biological systems by using such a chemosensing ensemble approach.

It is well known that thiols are the essential component of Cu^{2+}-dependent metalloenzymes, such as cytochrome *c* oxidase. Therefore, we may reasonable hypothesis that the interaction between thiols and Cu^{2+} receptor could lead to the liberation of the fluorophore, and thus the fluorescent turn ON chemosensor for the thiols detection could be achieved. Herein, a new NBD-based fluorescence ligand (**NP**) was designed and synthesized by a straightforward synthetic route. Notable fluorescence quenching was observed when Cu^{2+} ions were added into the solution of **NP**. In the presence of thiols, the fluorescence was significantly enhanced due to the detachment of Cu^{2+} ions of **NP**-Cu^{2+} ensemble. The new chemosensor was recyclable which was confirmed by the sequential treatment with Cu^{2+} ions and thiols. Confocal microscopy analysis was conducted to evaluate the levels of thiols in live cells.

2. Experimental

2.1. Reagents and instruments

Hydrazine hydrate, 7-nitrobenz-2-oxa-1,3-diazole chloride (NBD-Cl) and 2-hydroxy-1-naphthaldehyde were purchased from Sinopharm Chemical Reagent Co., Ltd. (China); unless otherwise noted, all reagents and solvents were of AR grade and used without further purification. Fresh stock solution of metal ions (nitrate salts, 20 mM), bioactive thiols and amino acids (20 mM) in H_2O were prepared for further experiments.

^{1}H-NMR and ^{13}C-NMR spectra were recorded with an AVANCE500MHZ spectrometer (BRUKER) with chemical shifts reported as *ppm* (in DMSO, TMS as internal standard). API mass spectra were recorded on a HP1100LC/MSD spectrometer. The elemental analyses of C, H, N were performed on a Vario EL III elemental analyzer. Fluorescence spectra were determined with LS 55 luminescence spectrometer (Perkin Elmer, USA). The absorption spectra measurements were measured with a Lambda 900 UV/VIS/NIR spectrophotometer (Perkin Elmer, USA). Fluorescent live cell images were recorded on an Olympus Fluoview FV 1000 IX81 inverted confocal laser-scanning microscope equipped with 405, 473, 559, and 635 nm laser diodes. All of the images were analysed by using Image J software version 1.44p.

2.2. General procedures of spectra detection

Stock solution of **NP** was prepared in HEPES aqueous buffer (THF:H_2O=3:7, 20 mM, pH=7.4). Excitation wavelength for **NP** was 420 nm. Before spectroscopic measurements, the solution was freshly prepared by diluting the high concentration stock solution. Each time a 3 mL solution of chemosensor was filled in a quartz cell of 1 cm optical path length, and different stock solutions of cations were added into the quartz cell gradually by using a microsyringe.

Stock solution of **NP**-Cu^{2+} for thiols detection was *in situ* prepared by the addition of 1.0 Eq. of Cu^{2+} to **NP** (10 μM) solution in HEPES buffer (THF:H_2O=5:5, 20 mM, pH=7.4). HRMS-ESI (positive mode, *m*/*z*) Calcd for [**NP**-Cu^{2+}+Na^+]$^+$, 782.0654, Found, 782.0659.

2.3. Quantum yield measurement

Fluorescence quantum yield was determined using optically matching solutions of rhodamine B (Φ_f=0.69 in ethanol) as standard at an excitation wavelength of 550 nm and the quantum yield is calculated using the equation [46]:

$$\Phi_{unk} = \Phi_{std}\frac{(F_{unk}/A_{unk})}{F_{std}/A_{std}}\left(\frac{\eta_{unk}}{\eta_{std}}\right)^2$$

Where Φ_{unk} and Φ_{std} are the radiative quantum yields of the sample and standard, F_{unk} and F_{std} are the integrated emission intensities of the corrected spectra for the sample and standard, A_{unk} and A_{std} are the absorbances of the sample and standard at the excitation wavelength, and η_{unk} and η_{std} are the indices of refraction of the sample and standard solutions, respectively. Excitation and emission slit widths were modified to adjust the luminescent intensity in a suitable range. All the spectroscopic measurements were performed at least in triplicate and averaged.

2.4. Association constant calculation

Generally, for the formation of 2:1 complexation species formed by the chemosensor compound and the guest cations, the Benesi–Hildebrand equation used is as follow [47]:

$$\frac{1}{F_0-F} = \frac{1}{K_a(F_0-F_{min})[Cu^{2+}]^2} + \frac{1}{F_0-F_{min}}$$

Where F and F_0 represent the fluorescence emission of **NP** in the presence and absence of Cu^{2+}, respectively, F_{min} is the saturated emission of **NP** in the presence of excess amount of Cu^{2+}; [Cu^{2+}] is the concentration of Cu^{2+} ion added, and K_a is the binding constant.

2.5. Synthesis and characterization of the fluorescent ligand (*NP*)

2.5.1. Synthesis and characterization of NBD-$NHNH_2$ (*1*)

7-nitrobenz-2-oxa-1,3-diazole chloride (NBD-Cl) (100 mg, 0.5 mmol, 1 Eq.) was dissolved in chloroform (50 mL). 1% hydrazine in 50 mL methanol was added into the above solution, and then the reaction mixture was stirred at room temperature for 1 h. A yellow-brown precipitate was formed and isolated without further purification in quantitative yield [48]. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.04 (d, 1H, 3J=10.5 Hz), 6.37 (d, 1H, 3J=10.5 Hz).

2.5.2. Synthesis and characterization of *NP*

In a 100 mL round-bottom flask, a solution of 2-hydroxy-1-naphthaldehyde (0.189 g, 1.1 mmol) in 10 mL ethanol was mixed with NBD-$NHNH_2$ (**1**) (0.195 g, 1 mmol) in 30 mL hot methanol. The reaction mixture was then heated to reflux for 8 h to form a brown black precipitate. After cooling to room temperature, the precipitate was filtered and washed with methanol for three times. The residue was purified by silica gel column chromatography using CH_2Cl_2/CH_3OH (v/v, 10:1) as the eluent. The fractions containing the target product were collected, and the solvent was evaporated. **NP** was then obtained with 67% yield. 1H NMR (DMSO-d_6, 500 MHz) δ(*ppm*): 12.88 (1H, s), 10.01 (1H, s), 9.61 (1H, d), 8.65 (1H, d), 8.04 (1H, d), 7.95 (1H, d), 7.89 (1H, m), 7.62 (H, m), 7.42 (H, m), 7.27 (1H, t). 13C NMR (DMSO-d_6, 125 MHz) δ(*ppm*): 160.6, 150.9, 146.8, 145.0, 144.4, 139.1, 129.6, 121.1, 112.4, 106.4, 103.9, 103.1, 97.9, 96.9. ES-API (negative mode, *m*/*z*) Calcd for $C_{17}H_{11}N_5O_4$: 349.08. Found: 348.1 [**NP**–H^+]$^-$. Anal. Calcd for: C, 58.45; H, 3.17; N, 20.05, Found: C, 58.28; H, 3.26; N, 20.27.

2.6. Fluorescence imaging of MDA-MB-231 cells with *NP*

For the fluorescence microscope imaging, MDA-MB-231 cells were typically seeded at a density of 5×10^4 cells/mL in a 22 mm cover-glass bottom culture dishes (ProSciTech, AU). After 24 h, the cells were washed with PBS buffer for three times, and then stained with **NP**-Cu^{2+} (10 μM) in PBS medium for 30 min at 37 °C.