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A novel NBD-based fluorescent turn-on probe for the detection of cysteine and homocysteine in living cells

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ABSTRACT

Biothiols, such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), are involved in a number of biological processes and play crucial roles in biological systems. Thus, the detection of biothiols is highly important for early diagnosis of diseases and evaluation of disease progression. Herein, we developed a new turn-on fluorescent probe **1** based on 7-nitro-2,1,3-benzoxadiazole (NBD) with high selectivity and sensitivity for Cys/Hcy on account of nucleophilic substitution and Smiles rearrangement reaction. The probe could sense Cys/Hcy rapidly, the intensity of fluorescence increased immediately within 1 min. Furthermore, the probe is low toxic and has been successfully applied to detect intracellular Cys/Hcy by cell fluorescence imaging in living normal and cancer cells.

1. Introduction

Biothiols, such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), are involved in a number of biological processes and play crucial roles in maintaining the appropriate redox status of biological systems. Cys and Hcy are essential biological molecules required for detoxifying function, immunological competence, as well as growth and delay of ageing of cells and tissues in living systems [1,2]. Abnormal levels of Cys have been associated with slow growth, hair depigmentation, liver damage, loss of muscle and fat, skin lesion and cancer etc. [3]. Homocysteine (Hcy) has been implicated in various types of vascular and renal diseases and was regarded as a risk factor for disorders of cardiovascular diseases and Alzheimer's disease [4,5]. Reduced glutathione (GSH) is the most abundant intracellular nonprotein thiol (1–10 mM) and a biomarker of oxidative stress [6,7]. It has been revealed that GSH plays a critical role in controlling oxidative stress in order to maintain the redox homeostasis for cell growth and function [8]. Aberrant levels of GSH have been correlated with various diseases, e.g. AIDS, cancer, liver damage and neurodegenerative diseases [9]. More importantly, many studies have shown that Cys/Hcy and GSH levels are interrelated inbiological systems [10–13]. GSH is contemplated as a putative intracellular reservoir of Cys in the liver of adult rats [14]. In addition, the synthesis of GSH is dependent on the trans-sulphuration of Hcy. Thus, the detection of biothiols is highly important for early diagnosis of diseases and evaluation of disease progression.

Among the reported detection methods, fluorescent sensing has attracted considerable attention due to their simplicity, low cost, selectivity and sensitivity. A large number of fluorescent probes have been developed in recent years to detect and sense these biologically important species [15–22].

The corresponding design strategies are generally based on the strong nucleophilicity of the thiol group combined with various mechanisms including Michael addition, cyclization reaction, displacement of coordination, cleavage reactions, and others [15–22]. A typical fluorescent probe is constructed by incorporating the specific recognition unit into a classic fluorophore platform. Various recognition units have been utilized to detect the biothiols, such as aldehydes, acrylates, 2,4-dinitrophenyl ester, 2,4-dinitrophenyl sulfonyl, methoxythiophenol and disulfide etc. [15–22]. Thereby, it is appreciated to discovery and optimize original recognition units as well as to construct novel fluorescent probes with improved performance. Due to the electrophilicity [23,24] and biocompatibility [25] of pyrimidine, we hypothesized that pyrimidiny-thioether has the potential function as a novel recognition unit to discriminate biothiols. To the best of our knowledge, vary recently a probe library for thiols containing pyrimidiny-ether was reported by Tang's group [26], the pyrimidiny-thioether has never been investigated to construct fluorescent probes. Hence, we speculated that a novel fluorescent probe candidate for recognition of biothios should be generated by incorporating pyrimidiny-thioether onto a fluorophore platform.

Owing to the good photochemical/photophysical properties and cell permeability, 7-nitro-2,1,3-benzoxadiazole (NBD) chromophore has been used for the design of fluorescent reagents [27–31]. Our group has considerable interest in the design and synthesis of NBD-based

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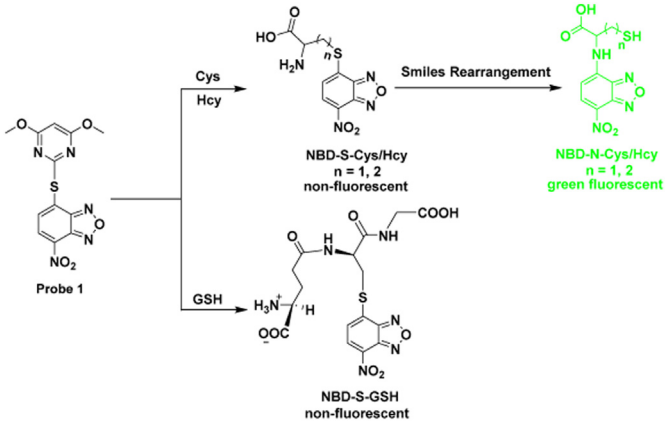


Scheme 1. Synthesis of probe **1**.

dyes as fluorescent probes [32–35]. To test the above hypothesis, we incorporated 4,6-dimethoxy-2-pyrimidiny-thioether onto NBD chromophore generated an NBD-based probe containing original recognition units (probe **1**) in this paper (Scheme 1). Probe **1** could discriminate Cys/Hcy from GSH based on the Smiles rearrangement reaction [36]. In view of the recommendable properties such as high specificity, turn-on fluorescent response and relatively low detection limit, probe **1** has been successfully applied for the fluorescence imaging of Cys/Hcy in living normal and cancer cells.

2. Experiment Section

2.1. Materials and Measurements

All reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Dimethyl sulfoxide (DMSO) in chromatographic purity and deionized water were used in detection. ^{1}H NMR spectra were recorded on a Varian Model Mercury 400 MHz spectrometer. ^{1}H NMR chemical shifts (δ) are given in ppm (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) downfield from Me_4Si, determined by $CDCl_3$ (δ = 7.26 ppm). ^{13}C NMR spectra were recorded on a Varian Model Mercury 100 MHz spectrometer. ^{13}C NMR chemical shifts (δ) are reported in ppm with the internal d_6-DMSO at δ 77.0 ppm as standard. Spectrometer UV–vis spectra were acquired on Shimadzu UV-2350 Spectrophotometer. Fluorescence spectra were measured by Varian Cary Eclipse fluorescence spectrophotometer. Electrospray ionization mass spectra (ESI-MS) were acquired with Agilent 1100 Series LC/MSD and AB SCIEX TripleTOFTM 5600+ mass spectrometer. All spectra were recorded at room temperature, except for the confocal laser scanning microscopic images.

The fluorescence quantum yields (Φ_f) of the probe **1** before and after reaction with Cys were calculated using the following relationship (Eq. 1):

$$\Phi_f = \Phi_{ref}\ F_{sampl}\ A_{ref}/F_{ref}\ A_{sampl} \quad (1)$$

Here, F denotes the integral of the corrected fluorescence spectrum, A is the absorbance at the excitation wavelength, ref. and sampl denote parameters from the reference and unknown experimental samples, respectively. The reference systems used was Rhodamine B (Φ_f = 0.86 in CH_3OH).

2.2. Procedure for Hcy and Cys Sensing

A stock solution of probe **1** (1 mM) was prepared in 100% CH_3CN and was subsequently diluted to prepare appropriate concentration solutions of probe **1** in CH_3CN/PBS buffer (3:7, v/v, 10 mM, pH 7.4) at 25 °C. Hcy (or Cys) stock solutions were freshly prepared prior to each experiment. Excitation was at 460 nm and emission was detected at 547 nm. The excitation and emission slit widths were set at 10 nm.

2.3. Detection Limits

The detection limit was calculated based on the fluorescence titration. In the absence of Hcy (or Cys), the fluorescence emission spectrum of probe **1** was measured by five times and the standard deviation of blank measurement was achieved. To gain the slop, the fluorescence intensity at 547 nm was plotted to the concentration of Hcy (or Cys). So the detection limit was calculated with the following Eq. (2):

$$\text{Detection limit} = 3\sigma/k \quad (2)$$

where σ is the standard deviation of blank measurement, k is the slop between the fluorescence intensity versus Hcy (or Cys) concentration.

2.4. MTT Assay

Living cells which cultured in DMEM medium supplemented with 10% fetal calf serum and incubated at 37 °C in 5% CO_2/air atmosphere seeded into 96-well plates, then 0, 5, 10, 15, 20 μM probe **1** in DMEM were added (n = 3), respectively. Untreated assay with DMEM (n = 3) was also conducted under the same conditions. Next, the cells were incubated at 37 °C for 12 h. Then, the media was discared and 10 μL of the MTT solution (5 mg/mL) was added to each well, followed by incubation at 37 °C for 4 h. The supernatant was abandoned and 100 μL of CH_3CN was added to each well. After shaking the plates for 10 min, optical density (OD) values of the wells were read with enzyme-labeled instrument at 490 nm. The cell viability rate (VR) was calculated according to the Eq. (3):

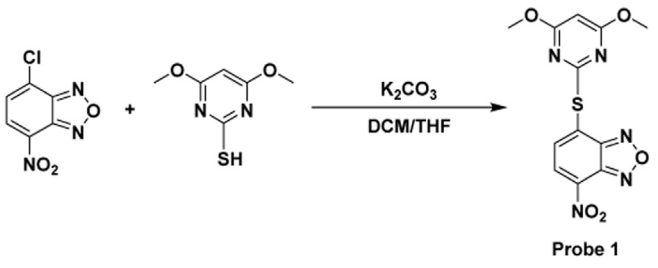
$$VR = (OD_1 - OD_0)/(OD_2 - OD_0) * 100\% \quad (3)$$

where OD_1 is the optical density of the experimental group, OD_2 is the optical density of the control group, and OD_0 is the optical density of untreated assay group.

2.5. Cell Culture and Living Cell Imaging

Living cells were incubated in DMEM with 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin at 37 °C in 5% CO_2. Living cells were seeded at a density of 5×10^4 cells per well (200 μL) in a 6-well plate.

The cells were imaged by using a confocal laser scanning microscope (λ_{ex} = 460 nm, ZEISS-LSM-710). Probe **1** (5 mM, 2 μL) in DMSO was added to living cells in a six-compartment cell culture plate that contained 1.0 mL culture medium, and was incubated at 37 °C for 20 min. After removing the culture medium and washing with PBS three times, the fluorescence images of cells were taken. For the control experiment, the cells in a six-compartment cell culture plate that contained 1.0 mL culture medium were treated with 5 mM Nethylmaleimide (NEM) in culture media for 30 min at 37 °C in a humidified incubator. After washing with PBS for three times, the cells



Scheme 2. Proposed sensing mechanisms of probe **1** for Cys and Hcy.