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Hydrazide D-luciferin for *in vitro* selective detection and intratumoral imaging of Cu^{2+}

Zhen Zheng [a], Lin Wang [b], Wei Tang [a], Peiyao Chen [a], Hui Zhu [a], Yue Yuan [a], Gongyu Li [a], Huafeng Zhang [b], Gaolin Liang [a,*]

[a] CAS Key Laboratory of Soft Matter Chemistry, Department of Chemistry, University of Science and Technology of China, Hefei, Anhui 230026, China
[b] School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China

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ABSTRACT

Copper is an essential micronutrient involved in fundamental life processes but using a bioluminescence (BL) probe to selectively sense Cu^{2+} *in vitro* or image Cu^{2+} *in vivo* is still unavailable. Herein, a latent BL probe hydrazide D-luciferin (**1**) was rationally designed and successfully applied it for selective detection of Cu^{2+} *in vitro* and imaging Cu^{2+} in living cells and in tumors. Upon the catalysis of Cu^{2+}, **1** was converted to D-luciferin and turned on the BL in the presence of firefly luciferase (fLuc). *In vitro* tests indicated that **1** could be applied for highly selective sensing Cu^{2+} within the range of 0–80 μM with a limit of detection (LOD) of 39.0 nM. Cell and animal experiments indicated that **1** could be applied for specific BL imaging of Cu^{2+} in living cells and tumors and the BL signal of **1** was more stable and longer than that of D-luciferin. We envision that this unique probe **1** might serve as an elucidative tool for further exploration of the biological roles of Cu^{2+} in physiological and pathological processes in the near future.

1. Introduction

Copper (Cu), one of the essential micronutrients, is involved in fundamental life processes conserved throughout all life forms (Keller et al., 2012). It serves as a cofactor for a variety of proteins and plays an important role in cellular metabolism (Fatemi et al., 2010; Rodriguez-Granillo et al., 2010). Recent studies suggested that many cancer types exhibit an increase of intratumoral copper and/or an altered systemic distribution of copper (Denoyer et al., 2015). The realization that Cu^{2+} is closely involved in physiological and pathological processes has greatly promoted the development of methods (or sensors) for Cu^{2+} detection/imaging. Traditional instrument-assisted techniques (*e.g.* inductively coupled plasma mass spectroscopy) are of high sensitivity for Cu^{2+} detection but are incapable of detecting Cu^{2+} in real-time due to their off-line feature (Becker et al., 2005; Zhang and Davison, 2000). Therefore, a variety of fluorescent probes have been developed for non-invasive detection or imaging of Cu^{2+} intracellularly (Jung et al., 2009; Yao et al., 2014; Xianyu et al., 2013). However, due to the paramagnetic nature of Cu^{2+}, most of the early-reported cation sensors, upon binding with Cu^{2+}, undergo fluorescence quenching (Chen et al., 2013). Thus, these fluorescence "turn-off" probes are not as sensitive as those fluorescence "turn-on" ones due to higher background signal (Wang et al., 2014; Mei et al., 2007). Nevertheless, current "turn-on" fluorescence probes for Cu^{2+} detection are limited in conjugation of fluorophores or quantum dots which suffer from poor water-solubility and biocompatibility, as well as strong auto fluorescence from the living body (Xu et al., 2009; Qu et al., 2012; Liu et al., 2011; Cai et al., 2014; Swamy et al., 2008).

Bioluminescent imaging (BLI) is a well-established, noninvasive imaging technique which has been widely applied in imaging various life processes (*e.g.*, cell proliferation and migration, gene expression, and enzyme activities) (Dragulescu-Andrasi et al., 2009; Cruz-Aguado et al., 2004; Wang et al., 2016; Kojima et al., 2013). Generally, BLI employs the decarboxylic catalysis of firefly luciferase (fLuc) on its substrate luciferin in the presence of adenosine triphosphate (ATP), O_2, and Mg^{2+}, emitting visible light in the process (Marques et al., 2009; McCutcheon et al., 2012; Conley et al., 2012). The generated bioluminescent photons can even penetrate tissues in intact rodents, rendering BLI well suitable for *in vivo* imaging (Yang et al., 2013; Van de Bittner et al., 2013). Moreover, compared with fluorescence techniques, BLI does not require external excitation and therefore exhibits higher signal-to-noise ratios. Due to the superiority of BLI, a variety of bioluminescence (BL) probes have been developed for efficient detection (or monitoring) of important analytes (Mofford et al., 2015; Takakura et al., 2015; Wu et al. 2014; Cohen et al., 2010; Yang et al., 2012). However, to the best of our knowledge, BLI-based probe for

* Corresponding author.
E-mail address: gliang@ustc.edu.cn (G. Liang).

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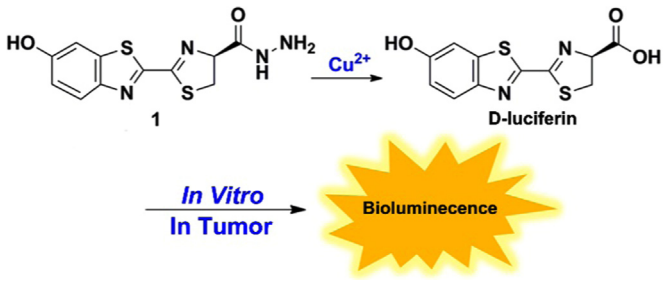

Scheme 1. Schematic illustration of Cu^{2+}-catalyzed transformation of 1 into D-luciferin for bioluminescence detection or imaging of Cu^{2+} *in vitro* and in tumor.

in vitro or *in vivo* imaging of Cu^{2+} is still unavailable.

Herein, we report a highly selective BL turn-on probe for Cu^{2+} detection *in vitro*, as well as its initial imaging applications in living cells and tumors. Since Cu^{2+} has been well recognized with its high specificity and affinity towards hydrazide structures and in turn promotes the hydrolysis of the hydrazide group (Dujols et al., 1997; Udhayakumari et al., 2014), we realized that the introduction of a hydrazide group on D-luciferin would confer a BLI probe for specific detection of Cu^{2+}. As outlined in Scheme 1, hydrazine was used to react with the carboxyl acid group of D-luciferin to yield the hydrazide D-luciferin (**1**) which is not the substrate for fLuc. Upon a Cu^{2+}-triggered hydrolysis (Scheme S1), the caged D-luciferin (*i.e.*, **1**) yields the active substrate free D-luciferin for luciferase to generate a light readout (Scheme 1).

2. Experimental

2.1. General methods

All the starting materials were obtained from Adamas or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All chemicals were reagent grade or better. 2-cyano-6-hydroxybenzothiazole (CHBT) was obtained from Shanghai Chemical Pharm-Intermediate Tech. Co. Firefly luciferase plasmid was purchased from Promega. ^{1}H NMR and ^{13}C NMR spectra were obtained on a 400 MHz Bruker AV 400 or 300 MHz Bruker AV 300. The spectra of electrospray ionization-mass spectrometry (ESI-MS) were recorded on a LCQ Advantage MAX ion trap mass spectrometer (Thermo Fisher). ESI-TOF/TOF mass spectra were obtained on a time-of-flight Agilent Technologies 6224 mass spectrometer. HPLC analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column with CH_3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluent. Luminescence emission spectra were obtained on a Hitachi F-4600 fluorescence spectrophotometer with the Xe lamp shut off. MDA-MB-231 cells were routinely cultured in Dul-becco's modified Eagle's medium (DMEM, Hycolon) supplemented with 10% fetal bovine serum at 37 °C, 5% CO2, and humid atmosphere. 4–6 week old (weighting 19–20 g) BALB/c nude mice were used for animal experiments. Bioluminescence values at different points for the cells in Fig. S15 were recorded on a Promega GloMax®-Multi.

2.2. Synthesis of D-luciferin

D-cysteine hydrochloride monohydrate (30.0 mg, 0.171 mM) and CHBT (28.7 mg, 0.16 mM) were suspended in MeOH: H_2O (2: 1, v/v, 10 mL) in a 20 mL vial. Potassium carbonate (22.7 mg, 0.16 mM) was then added to the mixture, and the resulting bright yellow-green solution was stirred under N_2 for 20 min Upon consumption of CHBT as evidenced by thin layer chromatograph analysis, the methanol was removed *in vacuo* and the remaining aqueous solution was acidified to pH 3 with 1 M HCl, and then D-luciferin precipitated. Pure D-luciferin was obtained after filtration and washing with water. ^{1}H NMR of D-luciferin (400 MHz, CD_3OD) δ (ppm): 7.90 (d, J=8.9 Hz, 1 H), 7.34 (d, J=2.2 Hz, 1 H), 7.06 (dd, J_1=8.9 Hz, J_2=2.4 Hz, 1 H), 5.38 (t, 1H), 3.76 (m, 2 H) (Fig. S1). MS: calculated for D-luciferin [$(M-H)^-$]: 278.99; obsevd. ESI-MS: *m/z* 279.02 (Fig. S2).

2.3. Synthesis of hydrazide-D-luciferin (1)

The isobutyl chloroformate (IBCF, 13.6 mg, 0.1 mmol) was added to a mixture of D-luciferin (28 mg, 0.1 mmol) and 4-methylmorpholine (MMP, 10.1 mg, 0.1 mmol) in CH_3OH (2 mL) at 0 °C and the reaction mixture was stirred for 30 min Hydrazine hydrate (7.3 mg, 0.15 mmol) was added to the reaction mixture and further stirred for 2 h at 0 °C then overnight at room temperature. The pure product **1** was obtained after HPLC purification. ^{1}H NMR of **1** (300 MHz, DMSO-d_6) δ (ppm): 10.2 (s, 1 H), 9.45 (s, 1 H), 7.96 (d, J=8.9 Hz, 2 H), 7.44 (d, J=2.2 Hz, 2 H), 7.06 (dd, J_1=8.9, J_2=2.3 Hz, 2 H), 5.21 (t, J=9.3 Hz, 1 H), 3.63 (m, 2 H) (Fig. S3). ^{13}C NMR of **1** (75 MHz, DMSO-d_6) δ (ppm): 168.3, 164.4, 157.3, 156.7, 146.2, 131.0, 124.8, 117.1, 106.7, 77.82, 34.43 (Fig. S4). MS: calc. [$(M+H)^+$]: 295.0323, obsvd. ESI-MS: *m/z* 295.0305 (Fig. S5).

2.4. Binding constant assay

The binding constant (K_s) assay study of probe **1** with Cu^{2+} was calculated according to calibration curve in Fig. S6. The nonlinear regression equation of fluorescent binding constant of probe **1** with Cu^{2+} was obtained with the equation below:

$$Y = Y_0 + \frac{Y_{lim} - Y_0}{2}\{1 + \frac{C_M}{C_L} + \frac{1}{K_s C_L} - [(1 + \frac{C_M}{C_L} + \frac{1}{K_s C_L})^2 - 4\frac{C_M}{C_L}]^{1/2}$$

where Y_0 is the original fluorescence intensity of probe **1** in the absence of Cu^{2+}. Y_{lim} is the limit value of the change in fluorescence. C_L is the concentration of probe **1**. C_M is the concentration of Cu^{2+} added.

2.5. Expression of firefly luciferase

BL 21 cells were transformed with the appropriate plasmids (pET28a-Luc) and cultured in LB medium supplemented with 50 μg/mL kanamycin at 37 °C until their OD_{600} reached 0.6–0.8. Then the cells were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16 °C for 20 h. The bacterial cells were precipitated (5000 × g, 20 min) and the pellets were suspended in lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.0) and sonicated to disrupt the bacterial cells on ice. The His-tagged enzymes were purified using Ni-NTA-agarose (Sangong) equilibrated in TBS buffer (50 mM Tris, 150 mM NaCl, pH 8.0) supplemented with 10 mM imidazole. After the enzyme supernatant was loaded in the Ni-NTA column, rotated for 1 h, and the column was washed with 10 times of column volume (CV) of TBS buffer (50 mM Tris, 150 mM NaCl, 10 mM imidazole, pH 8.0) and 5 times of CV of TBS buffer (50 mM Tris, 150 mM NaCl, pH 8.0, 50 mM imidazole), the enzyme was eluted out by adding 0.5 time of CV of TBS buffer (50 mM Tris, 150 mM NaCl, 250 mM imidazole, pH 8.0) for six times to the column. Each fraction of the column chromatograph was analyzed with SDS-PAGE. The protein collected from elution after dialysis was used without further purification (Fig. S7) and the enzyme concentration was determined with BCA method (Fig. S8).