Regenerative fluorescence “turn-on” probe for biothiols through Cu(II)/Cu(I) redox conversion

Kyung-Sik Lee, Jongmin Park, Hee-Jun Park, Young Keun Chung, Seung Bum Park, Hae-Jo Kim, Ik-Soo Shin, Jong-In Hong

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We present a regenerative molecular sensor for biothiols where a non-fluorescent copper(II) coumarin complex (CuII:CuL) probe turns to a strongly fluorescent Cu(I) complex by thiol-containing amino acids (Cys, Hcy, GSH). During the recognition process, Cu(I) ion of CuII:CuL undergoes chemical reduction followed by substitution of the coordinating ligands with the biothiols. Interestingly, the strongly fluorescent Cu(I) complex, which quantifies the amount of the target biothiol, was oxidized back to its original CuII:CuL by chemical (or electrochemical) oxidation, and could be repeatedly reused as a thiol probe for several turn-overs. CuII:CuL was successfully applied in fluorescent imaging of the cellular GSH.

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1. Introduction

Biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are known to play an important role in a myriad of vital cellular processes, including redox homeostasis [1,2] and cellular growth [3,4]. Alterations in the cellular levels of biothiols are linked to various chronic diseases such as arthritis, cancer, and HIV/AIDS [5,6]. Considerable attention has been paid to the detection of biothiols using analytical techniques such as mass spectrometry [7], gas chromatography [8], and high-performance liquid chromatography [9] coupled with spectrophotometric or electrochemical methods [10,11]. Recently, fluorescent probes have been used for detecting biothiols due to their simplicity, high selectivity, and good sensitivity [12,13]. Most of fluorescent methods, however, are based on irreversible chemical reactions between the probe and the thiol providing only a single-use assay: Michael addition [14–17], cyclization with aldehyde [18,19], or a cleavage of sulfur-containing bond by the thiol [20].

Herein, we report a unique molecular probe for biothiol detection, wherein the probe undergoes a series of chemical reactions during target recognition (Scheme 1). A synthetic probe, copper(II) coumarin complex (CuII:CuL), obtained by combining an equimolar mixture of CuCl2 and the ligand (L), selectively binds to biothiols, generating strong fluorescence which is caused by the chemical reduction of Cu(II) into Cu(I) during the binding event. Interestingly, Cu(I) ion can be oxidized to Cu(II) ion by the addition of N-ethylmaleimide (NEM), which completely quenches the fluorescence again, and this fluorescence “off/on” cycle is observed repeatedly for several turn-overs without a significant decrease in the fluorescence intensity.

2. Experimental

2.1. Materials and instruments

Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Chromatography was carried out on silica gel 60 (230–400 mesh ASTM; Merck). Thin layer chromatography (TLC) was carried out using Merck 60 F254 alumina plates with a thickness of 0.25 mm. 1H NMR and 13C NMR spectra were recorded using Bruker 300 or Varian 200. Mass spectra were obtained using a JMS-HX 110A/110A Tandem Mass Spectrometer (JEOL). UV absorption spectra were obtained on Agilent 8453 Double Beam UV/VIS Spectrometer. Flu-
Spectrofluorometer.

2.2. Synthetic procedure

2.2.1. Synthesis of ligand

To a solution of coumarin aldehyde [21] (200 mg, 1.05 mmol) in EtOAc (10 mL) was added 2-picolylamine (120 mg, 1.1 mmol). Resulting clear yellow solution was further stirred at room temp. for 1 h to afford yellow precipitates, which were filtered off, washed with cold EtOAc, and dried in air to obtain the imine intermediate. The yellow solid was further reduced by sodium triacetoxyborohydride without further purification. To a solution of the intermediate (200 mg, 0.71 mmol) in CH$_2$Cl$_2$ (10 mL) was added NaHBO$_2$(OAc)$_3$, (190 mg, 0.89 mmol). Resulting clear yellow solution was further stirred at room temp. for 12 h, which was purified by column chromatography using dichloromethane/MeOH (10:1 v/v, $R_f$ = 0.40) as an eluent. Evaporation and drying in vacuum afforded the desired product in 85% yield over 2 steps. $^1$H NMR (DMSO-d$_6$, 300 MHz): $\delta$/ppm 9.08 (s, 1H), 7.90 (d, $J$=9.02 Hz, 1H), 7.77 (s, 1H), 7.42 (d, $^3$J=6.85 Hz, 2H), 7.28 (s, 1H), 6.71 (d, $J$=7.98 Hz, 1H), 6.15 (d, $J$=8.99 Hz, 1H), 4.07 (s, 2H), 3.89 (s, 2H). $^{13}$C NMR (DMSO-d$_6$, 75 MHz): $\delta$/ppm 163.26, 160.84, 158.54, 153.44, 149.38, 145.53, 137.19, 128.61, 122.81, 122.72, 113.87, 110.96, 110.74, 110.40, 53.35, 42.90. HRMS (FAB+, m-NBA): $m/z$ obs'd 283.1088 ([M+H]$,^+$, 283.1083 cal'd for C$_{16}$H$_{14}$N$_2$O$_3$).

2.2.2. Synthesis of Cu$^{II}$L (probe 1)

To a solution of free ligand (38 mg, 0.10 mmol) in 1 mL CH$_3$CN was added CuCl$_2$ (0.10 mmol) in 1 mL MeOH. Resulting blue solution was further stirred for 1 h at room temp. to afford a light blue precipitates, which were filtered off, washed with CH$_3$CN (2 × 1 mL) and dried in vacuum. Yield 70% (Scheme 2). MS (ESI, positive): $m/z$ obs'd 344.02 (cal'd 344.02 for [M–Cl]$^+$).

Single crystals of Cu$^{II}$L suitable for the X-ray crystallography were obtained by slow evaporation of diethylether into a MeOH/water solution of Cu$^{II}$L at room temperature. Diffraction data were collected on a Bruker SMART X-ray diffractometer at room temperature using graphite-monochromated Mo-K$_\alpha$ radiation ($\lambda$ = 0.71073 Å). The structures were solved by direct methods (SHELXS-97), and refined against all $F^2$ data (SHELX-97). All non-hydrogen atoms were refined with anisotropic thermal parameters and the hydrogen atoms were treated as idealized contributions. Crystallographic data in CIF format for Cu$^{II}$L is contained in CCDC No. 1013353.

2.3. Preparation for fluorescence study

A stock solution (10 mM) of Cu$^{II}$L in DMSO was prepared and used by dilution in aqueous DMSO solution for in vitro and in vivo fluorescence experiments. In a typical experiment, test solutions were prepared by placing 2 μL of the probe stock solution into a test tube, adding an appropriate amount of each amino acid, and diluting the solution to 2 mL with buffer (0.10 M HEPES, pH 7.4). Normally, excitation was at 325 nm. Both the excitation and emission slit widths were 3 nm × 3 nm. Fluorescence spectra were monitored after addition of amino acids.

2.4. Fluorescence cell imaging of HeLa cells

For the detection of biothiols in live cells, HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum. The cells were seeded on a Ø 35 mm glass-bottomed dish at a density of 1 × 10$^5$ cells in a culture medium overnight for live-cell imaging by confocal laser scanning microscopy (CLSM). The HeLa cells were treated with 10 μM of Cu$^{II}$L in 2 mL of serum free medium for 0.5 h and washed with 3 times with pre-warmed 1 × PBS before imaging by CLSM.

2.5. Electrochemistry

Electrochemical studies were performed using a CHInstruments 650B Electrochemical Analyzer (CH Instruments, Inc., Texas, USA). All electrochemical studies were referenced with respect to an Ag/AgCl (sat’d with KCl) reference electrode. A glassy carbon disk.
3.2. X-ray structure of probe

In our approach, Cu(II) ion in the synthetic probe (I, CuII) serves as a fluorescent quencher for the coumarin unit [22–24]. At the beginning, we assumed that Cu(II) ion would interact not only with nitrogens of the 2-picolylamine unit, but also with the hydroxyl oxygen of the 7-hydroxycoumarin moiety since the oxygen atom might also donate its lone-pair of electrons along with the two nitrogen atoms in the coumarin moiety to Cu(II). It was expected that Cu(II) might withdraw electron density from the oxygen atom in the complex, thereby quenching fluorescence from the coumarin unit. However, the crystal structure of CuII revealed that the OH group in CuII did not coordinate with Cu(II) (Fig. 1). CuII was crystallized as a dimer by slow evaporation of diethyl ether into a methanol/water solution of CuII. The strong reduction in fluorescence of CuII was likely due to the paramagnetic character of Cu(II) alone [25–28]; fluorescence quenching takes places through photoinduced electron transfer from the coumarin singlet excited state to the bound Cu(II) ion.

3.2. PL, UV, NMR study

While CuII exhibits very low photoluminescence (PL) intensity, a large enhancement in the fluorescence emission was observed by the addition of biothiols. As shown in Fig. 2a, the emission intensity at 450 nm increased more than 100-fold compared to CuII itself, and was saturated at 30 equiv of GSH. The peak intensities exhibited a linear correlation with GSH concentrations up to 5 equiv of GSH ($R^2 = 0.997, y = 64.9x + 49.0, LOD = 15 \mu M$). Competitive binding experiments were performed in HEPES buffer (10 mM, pH 7.4) to determine the selectivity of CuII towards Cys among various natural amino acids. As displayed in Fig. 2b, only Cys showed a dramatic increase ($F/F_0 = 106$) [29] in the fluorescence intensity compared to other amino acids. Amino acids possessing acidic or basic side chains induced no significant changes in the fluorescence intensity except for His which showed $>$60% increase in the emission intensity compared to that upon the addition of Cys. (Fig. S3). The competitive experiments revealed that the fluorescence intensity of CuII was restored to levels similar to that of CuII-Cys adduct when Cys was added to the mixtures of CuII and other amino acids. Other biothiols such as Hcy and GSH also exhibited similar enhancements in the fluorescence intensity with $F/F_0 = 102$ and 94, respectively (Fig. S3). The fluorescence experiments indicated...
that CuII-L selectively reacted with amino acids containing a thiol side chain. Also, the prominent changes in the fluorescence intensity could even be observed by the naked eyes. Upon the addition of Cys, the solution mixture displayed strong fluorescence under a portable UV spectroscope, in contrast to the other amino acids (Fig. S4).

It was interesting to observe that the color of the solution began to change along with fluorescence enhancement during the addition of biothiol. The CuII-L solution originally had pale green color due to the presence of Cu(II) ion. It, however, quickly turned colorless after an excessive addition of biothiols, which implied that a conversion of Cu(II) to Cu(I) had occurred in the solution mixture. Since no precipitate of copper was observed, the only possible explanation could be the reduction of Cu(II) to Cu(I) ion by the thiol group. The electrospray mass spectrometric analysis confirmed that two biothiol molecules were definitely coordinated to one molecular probe, and copper was still located on the final adduct after the binding (Fig. S5). Therefore, three molecules of biothiols were expected to participate in the entire binding event in which one biothiol reduced Cu(II) to Cu(I), while the other two were coordinated to Cu(I).

The 1H NMR spectra strongly supports the conversion of Cu(II) to Cu(I) (Fig. S6). While CuII-L exhibited no proton signals in the 1H NMR spectrum due to the paramagnetic Cu(II), the binding adduct displayed definite proton signals owing to the formation of diamagnetic Cu(I).

3.3. Reusability study as a thiol probe

The electrochemical study also supports the electrochemical conversion of Cu(II) to Cu(I). In differential pulse voltammograms, a solution mixture of 1 mM CuII-L and 5 mM biothiol revealed only the characteristics of Cu(I)/Cu(II) oxidative transition at ~0.15 V (vs. Ag/AgCl), while CuII-L showed no oxidative peak (Fig. S7) [30,31]. Considering that the formal potential of thiol/dissulfide conversion is E° ~ ~0.25 V (vs. NHE) [32–35], one can expect that the Cu(II) ion in CuII-L could be readily reduced to Cu(I) ion by the thiol group. That is, Cu(II) can also be regenerated from Cu(I) by electrochemical oxidation. We carried out bulk electrolysis on the mixed solution of CuII-L and excessive N-acetyl cysteine (NAC), which is a cell-permeable model compound of biothiols, in order to re-oxidize Cu(I) of the binding adduct. After bulk electrolysis under ~0.15 V, the absorption band around 648 nm, which presumably comes from the Cu(I) ion, began to recover up to ~60%, and the emission revived with relatively poor recovery at 450 nm (Fig. S8). As shown in Fig. 3a, upon successive application of biothiols and oxidative potential, the fluorescence “off/on” cycle was partly repetitive over several turn-overs, although the recovery of the emission intensity significantly decreases over time. The absorption at 648 nm was mainly derived from Cu(II) ion, while the fluorescence came from the coumarin unit. We think that the relatively poor recovery of fluorescence emission, compared to that of absorption, could be due to electrochemical instability of the binding complex. Currently, efforts are underway to obtain a better condition for electrochemical reproducibility.

Compared to the application of the oxidative potential, CuII-L exhibited regenerative fluorescence ‘off/on’ upon the successive addition of NEM and NAC (Figs. 3b and c). NEM is known both as a good thiol scavenger via Michael addition and as an oxidizing agent in biological and non-biological processes. Therefore, NEM is expected to expel biothiols from CuII-L, the binding adduct while simultaneously re-oxidizing Cu(I) in CuII-L. As expected, the fluorescence of CuII-L significantly increased upon the addition of 10 equiv. NAC, but the subsequent addition of 10 equiv. NEM quenched the fluorescence completely. Unlike bulk electrolysis, these fluorescence ‘off/on’ cycles were repeatedly observed for more than three turn-overs. Although the initial fluorescence recovery was ~60%, no significant decrease was observed for later cycles. The 60% emission intensity recovery rate was almost same as that of absorption, which indicates that the transformation between Cu(II) and Cu(I) was the major factor for fluorescent sensing of biothiols. Furthermore, we checked changes in the absorption intensities of 100 μM CuII-L (probe 1) solution (10 mM HEPES, pH 7.4) upon the successive addition of NAC and NEM (Fig. S9). The absorption intensities at 648 nm indicate regenerative sensing ability of probe 1. Therefore, we assume that the Cu(II) ion was reduced to the Cu(I) ion by a biothiol, and two biothiols were additionally coordinated to Cu(I), resulting in the enhanced fluorescence emission (Scheme 1).
3.4. Cell study

CuII was further applied for cellular imaging of GSH, the most abundant cellular biothiol (Fig. 4) [36,37]. For the detection of cellular GSH levels, HeLa cells were treated with 10 μM CuII for 0.5 h and washed 3 times with PBS. The images of the cells were obtained using fluorescence microscopy. When the cells incubated with CuII were treated with 250 μM lipoic acid for 48 h to boost the cellular GSH level [38,39], the fluorescence intensity significantly increased compared to the control. In contrast, the fluorescence intensity decreased upon treatment with 1 mM NEM, a scavenger of GSH [40], for 30 min due to partial sequestering of GSH.

4. Conclusion

In conclusion, we demonstrated a new form of fluorescent probe for biothiols. The synthetic probe, CuII, exhibited a selective and significant fluorescence turn-on response toward biothiols over other natural amino acids in aqueous buffer (pH 7.4). This was attributable to the reduction of Cu(II) ion of CuII to Cu(I) in the binding complex. Interestingly, the redox-based mechanism presents the possibility for regenerative sensing of biothiols [41,42] in which the fluorescence emission could be recovered repeatedly by not only addition of NEM, but also application of the oxidative potential. Additionally, CuII was used successfully for the fluorescence imaging of the cellular GSH.

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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.snb.2016.06.089.

References


The fluorescence intensity of Cu^II-L-Cys adduct was about 89% of that of free ligand indicating that the coordinated oxygen of the ligand was released from 1 (see Fig. S3, ES). [29] M. Jayalakshmi, K. Balasubramanian, Int. J. Electrochem. Sci. 3 (2008) 1277.

Note that the standard reduction potential of Ag/AgCl (saturated with KCl) reference electrode employed here $E^o = +0.197$ V (vs. N.H.E.), so that the thiol/disulfide conversion is assumed to $E^o \approx -0.45$ V (vs. N.H.E.), which is highly negative enough to reduce Cu(II) into Cu(I).


Biographies

Dr. Kyung-Sik Lee obtained B.S. (2003) and M.S. (2005) in chemistry from Andong National University, Korea. He received his Ph.D. (2011), and was a postdoctoral fellow at Seoul National University under the supervision of Prof. J.-I. Hong (2011–2012). Currently he is working as a senior research engineer at the Hansol Chemical Co., Ltd. His research interests include the development of precursors for semiconductor manufacturing and the development of self-assembled superstructures based on metal-ligand.

Dr. Jongmin Park obtained his B.S. (2005) in chemistry and biological science from Seoul National University, Korea. He received his Ph.D. (2012) under the supervision of Prof. Seung Bum Park. Currently, he is working as a post-doctoral research fellow under guidance of Prof. Hakho Lee and Prof. Ralph Weissleder at Harvard Medical School, USA. His research interest is focused on discovery of bioactive small molecules, target identification of bioactive small molecules, and development of disease diagnostic tools with liquid biopsy strategy in clinic.

Hee-Jun Park is Ph.D. student in the Department of Chemistry, Seoul National University, Korea. His research interests include organometallic-based molecular probes using organic and inorganic multidisciplinary chemical science.

Dr. Young Keun Chung obtained his Ph.D. from Brown University, and did his postdoctoral work at University of California, Santa Barbara. He has been a professor at Seoul National University since 1987. He has published over 280 scientific papers. His research interests include development of new catalysts and new catalytic systems in relation to carboxylative cycloaddition and hydrogenation.

Dr. Seung Bum Park obtained his Ph.D. from Texas A&M University (2001) and did his postdoctoral training at Harvard University under the guidance of Prof. Stuart L. Schreiber. He started his independent career at Chemistry Department of Seoul National University in 2004. He is currently a full professor and director of CRI Center for Chemical Proteomics at SNU. He has published over 130 scientific papers and 30 patents. His research interests include chemical biology, molecular diversity, fluorescence sensors, high content screening, drug discovery, and target identification.

Dr. Ha-Jo Kim received his B.S. degree in Chemistry Education, and M.S. and Ph.D. degrees in Chemistry from Seoul National University, Korea with Professor Jong-In Hong (2002). After completing his postdoctoral research at University of Toronto, Canada with Professor Jik-Chin (2005), he joined Kyunggi University, Korea as a faculty member (2006). He is currently associate professor and chairperson of the Department of Chemistry at Hankuk University of Foreign Studies, Korea. His research focuses on fluorescent molecular probes for tumor imaging and therapy.

Dr. Ik-Soo Shin was born in 1975 in Korea, and obtained Ph.D. degree from Seoul National University (SNU) in 2007. He was a postdoctoral fellow at the University of Texas at Austin, and SNU. Currently he is an assistant professor at Soongsil University, in Korea. His research interests include the design and development of biosensors based on electrochemical method.

Dr. Jong-In Hong obtained his Ph.D. from Columbia University, and did his postdoctoral work at MIT. He has been a professor at Seoul National University since 1993. He has published over 170 scientific papers. His research interests include development of optical and/or electrochemical sensors for biologically or clinically important ions and molecules.