A selective and sensitive near-infrared fluorescent probe for real-time detection of Cu(i)†

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The disruption of copper homeostasis (Cu+/Cu2+) may cause neurodegenerative disorders. Thus, the need for understanding the role of Cu+ in physiological and pathological processes prompted the development of improved methods of Cu+ analysis. Herein, a new near-infrared (NIR) fluorescent turn-on probe (NPCu) for the detection of Cu+ was developed based on a Cu+-mediated benzylic ether bond cleavage mechanism. The probe showed high selectivity and sensitivity toward Cu+, and was successfully applied for bioimaging of Cu+ in living cells.

Introduction

As an essential trace transition metal element, found in both the oxidized Cu2+ and reduced Cu+ states in living organisms, copper is considered a vital redox-active cofactor for various cytosolic, mitochondrial and vesicular oxygen-processing enzymes, including cytochrome-c-oxidase,1-5 copper/zinc superoxide dismutase6 and metallothionein.7 The disproportionation of Cu+ in cells could produce reactive oxygen species (ROS), leading to oxidative damage of proteins, nucleic acids and lipids.8-10 There is a growing body of evidence to suggest that the imbalance of Cu+ may cause neurodegenerative disorders, such as prion, Parkinson’s, Alzheimer’s, Menkes, and Wilson’s diseases and amyotrophic lateral sclerosis.5,11 In addition, copper was also demonstrated to play a critical role in other pathological processes promoted the development of methods in Cu+ analysis. There are existing techniques such as electrochemistry,14-17 chromatography,18-21 pulse polarography,22 voltammetry23 and atomic absorption spectrophotometry (AAS)24,25 but these generally require sophisticated procedures and expensive instrumentation. More importantly, these methods cannot provide the real-time visualization of labile Cu+ in situ. Optical imaging techniques are non-invasive, highly sensitive, easily handled, and suitable for detecting analytes in biological system. In the past decade, a number of fluorescent probes have been designed for copper detection.26-30 Among this collection, NIR fluorescent probes stand out due to their unique properties of high penetration through tissues,31-34 low auto-fluorescence and less photodamage.35,36 T. Govindarajua et al. reported on the development of a NIR fluorescent probe with a tripicolylamine (N4) functionality for the detection of intracellular Cu+37 B. R. Cho et al. and W. Wan et al. developed probes with the recognition group being bis(2-((2-(ethylthio)ethyl)-thio)ethyl) amine (BETA) containing electron rich S atoms.38,39 Although these probes offer a promising strategy for intra-vital non-invasive quantitative imaging, they cannot completely discriminate Cu+ from other interfering cations, such as Cu2+, Co2+ or Hg2+. Developing a highly selective tool to detect Cu+, especially to visualize dynamic process of Cu+ in living system, is a challenging endeavour.

Herein, we developed a new near-infrared (NIR) fluorescent turn-on probe (NPCu) for the detection of Cu+, based on the mechanism of a Cu(i)-mediated benzylic ether bond cleavage. Our results demonstrate that the NPCu probe can effectively distinguish Cu+ from most interfering cations in vitro and in vivo.

Experimental

Materials and instruments

All reagents were purchased from commercial suppliers and used without further purification (Adamas-beta® or Lab Network), unless otherwise noted. All chemicals and solvents were used without purification unless otherwise noted. Column chromatography was carried out on silica gel (200–300 mesh)...
using an eluent of ethyl acetate and cyclohexane, dichloromethane/methanol, or ethyl acetate/methanol. TLC analyses were conducted on silica gel plates and visualized using UV (254 nm) and 360 nm on a Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz respectively. Melting points were determined on a Mel-Temp apparatus and were not corrected. UV/vis spectra were recorded on a UV-1800 (240 nm) spectrophotometer and fluorescence spectra were recorded on a FL3C-iHR320 spectrophotometer (HORIBA Instrument Inc.) Absorption and emission were measured using a 1 cm path length quartz cells. Fluorescence imaging was carried out with Nikon Ti-E laser scanning microscope that excited at 560 nm and an emission wavelength of 710 nm. The molar absorption coefficient of probe NPCu without Cu⁺ was calculated as ε = 1.76 × 10⁴ M⁻¹ cm⁻¹ while with Cu⁺ was 0.76 × 10⁴ M⁻¹ cm⁻¹. NPCu after Cu⁺ treatment exhibited a high quantum yield (φ = 0.39). The quantum yields of NPCu was 0.024 in aqueous medium when excited at the λmax (560 nm, ε = 0.76 × 10⁴ M⁻¹ cm⁻¹).

Cell cytotoxicity and imaging
Human alveolar epithelial A549 cells were grown in RPMI-1640 medium, containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, at 37 °C in a humidified incubator with an atmosphere of 5% CO₂/95% air. One day before the experiment, a 6-well culture plate was inoculated with logarithmic growth phase cells. The cells in the experimental group were incubated with Cu⁺ (50 μM) for 6 h. In the control group, only medium was added. After that, cells were incubated with NPCu (10 μM) at 37 °C for 1 h. Finally, an inverted microscope was used to observe the cells with DIC and Cy-3 fluorescence filters.

Measurement of absorption and fluorescence
For UV/vis and fluorescence titrations stock solution of NPCu probe were prepared (ε = 10 mM) in 25 mM PBS buffer (pH = 7.2). The solutions of cations were freshly prepared in PBS buffer solution. Working solutions of the probe and metal ions were prepared from the stock solutions. Excitation and emission spectra were carried out at 560 nm and 710 nm for NPCu with 4 nm slit widths. All buffers for pH titration were prepared in deionized water and adjusted to a suitable pH with HCl or NaOH aqueous solutions.

Selectivity of probes to Cu(i)
Each metal cation was diluted with PBS buffer solution to a 1 mM working solution for use, and mixed with 2 mL of NPCu solution (100 μM) in equal volume; in experiment 2, each metal cation was mixed with Cu(i), and each was configured. A mixed solution of 1 mM ion concentration was mixed with 2 mL of NPCu solution (50 μM) in equal volume. The samples from both experiments were incubated for 1 hour in a dark shaker under a 37 °C constant temperature shaker to determine the fluorescence intensity of each ion solution at an excitation wavelength of 560 nm and an emission wavelength of 710 nm. The molar absorption coefficients of probe NPCu without Cu⁺ was calculated as ε = 1.76 × 10⁴ M⁻¹ cm⁻¹ while with Cu⁺ was 0.76 × 10⁴ M⁻¹ cm⁻¹. NPCu after Cu⁺ treatment exhibited a high quantum yield (φ = 0.39). The quantum yields of NPCu was 0.024 in aqueous medium when excited at the λmax (560 nm, ε = 0.76 × 10⁴ M⁻¹ cm⁻¹).

Synthesis of the NPCu probe
To a mixture of compound 5 (297.5 mg, 0.7 mmol) and 3-1 (517.7 mg, 1.8 mmol) in 4 mL of anhydrous ethanol was added sodium acetate (119.6 mg, 1.46 mmol) in 2 mL of ethanol under a nitrogen atmosphere.

\[ \text{NPCu} \]

Scheme 1 Synthesis procedure of probe NPCu.
Results and discussion

Sensitivity research

We first evaluated its spectral properties and determined its responsiveness towards Cu(I). As shown in Fig. 1, the absorbance, extraction and emission spectra of NPCu peaked at 415 nm, 430 nm, and 560 nm respectively in 25 mM PBS (pH 7.2) containing 2 mM glutathione (GSH). The addition of Cu+ (10 eq.) induced significant signal changes in the optical properties of NPCu solutions. As shown in Fig. 1A, a marked redshift (~150 nm) was noted in absorption. Meanwhile, in the emission spectra, a turn-on response from 560 nm to 710 nm was noted upon introduction of Cu+ to the probe. After the incubation with Cu+, large Stokes shifts (>150 nm) both in the excitation and emission spectra were observed (Fig. 1B and C). This is considered a highly desirable feature as it increases the signal-to-noise ratio.

To investigate whether NPCu had the sensitivity to measure small fluctuations in Cu+ level in aqueous solutions, the probe (50 μM) was exposed to a range of Cu+ concentrations. The fluorescence intensity at 710 nm was plotted and exhibited good linear correlation ($R^2 = 0.995$) with Cu(I) levels from 0 to 1000 μM. A typical calibration curve is shown in Fig. 2 (inset). The detection limit for Cu+ was $9.1 \times 10^{-5}$ M ($S/N = 3$). It was shown that the fluorescence intensity of the probe progressively increased with increasing concentration of Cu+.

Fluorescence intensity changes of NPCu with time

To 2 mL of NPCu solution (50 μM) was added the same volume of Cu+ (1000 μM) at various time points. The excitation wavelength ($\lambda_{exc} = 560$ nm), 25 mM PBS (pH 7.2) containing 2 mM GSH. Fig. 3 Change of fluorescent intensity: NPCu (50 μM) reacted with Cu+ (1000 μM) at various time points. $\lambda_{exc}/\lambda_{em} = 560/710$ nm, 25 mM PBS (pH 7.2) containing 2 mM GSH.

Selectivity research

NPCu can be conveniently used as a ‘switch-on’ probe for the detection of Cu+ without interference from pH-related effects in physiological environments. As shown in Fig. 4, the specificity...
of NPCu ion detection was tested with various metal ions including Cu^{2+}, Co^{2+}, Hg^{2+}, and Fe^{3+}. NPCu exhibited a remarkable 30-fold enhancement in the NIR region ($\lambda_{ex} = 560$ nm) upon addition of Cu$^+$ (10 eq.) after 2 h, whereas the responses toward other ions were negligible. The NPCu exhibited high selectivity for Cu$^+$ over other biologically relevant interfering metals, including Cu$^{2+}$, Co$^{2+}$, Hg$^{2+}$, and Fe$^{3+}$.

Fluorimetric pH titration of NPCu

We further assessed the probe’s capability of detecting Cu$^+$ at different pH’s. The pH was adjusted between 4.0 and 10.0 by adding 1 M HCl, and the fluorescence titration was performed in a quartz colorimetric dish with a path length of 1 cm (constant temperature set to 25 °C). The absorption spectra (Fig. S2†) were collected from 200–410 nm and the fluorescence intensity was measured at 710 nm with excitation at 560 nm. In the absence of Cu$^+$, there was almost no change fluorescence intensity in the pH range of 4–10, which suggested this probe has considerable stability. Under neutral to mildly alkaline conditions, NPCu can maintain high responsiveness to Cu$^+$.

Sensing mechanisms

According to the structural characteristics of NPCu and literature reports, we speculate a new approach for the detection of Cu$^+$ in live cells through the development of a NIR probe (NPCu), which is comprised of two discrete elements: a NIR core (Fig. 5, black), a Cu$^+$ reactive moiety (Fig. 5, red). NPCu with a tetradentate ligand N$_2$O moiety, which is first employed as a highly selective trigger for the detection of Cu$^+$. Upon binding to Cu$^+$, the benzylic ether bond (C-O) of NPCu was cleaved, and cyanine-quinone dye was released from NPCu, leading to a robust NIR fluorescence enhancement. This mechanism was confirmed by mass spectrometry (Fig. S4†).

We also used orbital theory to better verify our hypothesis. LUMO and HOMO energies of NPCu were calculated to be $-3.55$ eV and $-1.91$ eV while the energies of NPCu$^+$ were $-3.37$ eV and $-1.02$ eV. The energy gaps (LUMO−HOMO) of NPCu and NPCu$^+$ are 2.45 eV and 1.64 eV, respectively. It is worth noting that after reacting with Cu$^+$, the UV absorption peak of NPCu was observed to have redshifted. Theoretical predictions were consistent with the spectroscopy experimental data, indicating that the proposed reaction mechanisms were reasonable.

Cytotoxicity and cell imaging

The cytotoxicity of NPCu was evaluated using the methylthiazolylidiphenyltetrazolium (MTT) viability assay. As indicated in Fig. S4†, A549 cells incubated with NPCu showed little decrease in cell viability. Viability was >70% at 30 µM NPCu, which indicates that NPCu is suitable for bioimaging applications at 10 µM. Next, we tested the utility of this probe for detecting Cu$^+$ in cultured A549 cells by confocal microscopy. The results clearly demonstrate that NPCu is permeable to membrane, and can react with intracellular Cu$^+$ to release near infrared fluorescent Cy-quinone dye in living cells (Fig. 7).

Conclusions

In summary, we have developed a new NIR fluorescent turn-on Cu$^+$ probe based on the cyanine-quinone fluorophore and novel Cu$^+$ receptor N$_2$O-ol skeleton. The probe NPCu exhibited high
sensitivity, good selectivity, and considerable stability at physiological pH. More importantly, NPCu showed excellent biocompatibility and high permeability for penetrating cell membrane and tracking intracellular Cu’. NPCu shows promising properties for non-invasive imaging of Cu’ in living cells and provides a useful tool to better understand the contribution of Cu’ in living systems.

Conflicts of interest

There are no conflicts to declare.

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