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An efficient two-photon ratiometric fluorescent probe platform for dual-channel imaging of lysosomes in living cells and tissues

Liyi Zhou[a,*], Dan-Qing Lu[b], Qianqian Wang[b], Shunqin Hu[a], Haifei Wang[a], Hongyan Sun[c], Xiaobing Zhang[b]

[a] College of Life Science and Chemistry, Hunan University of Technology, Hunan 412007, PR China
[b] Molecular Sciences and Biomedicine Laboratory, State Key Laboratory for Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Collaborative Innovation Center for Chemistry and Molecular Medicine, Hunan University, Changsha 410082, PR China
[c] Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, China

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ABSTRACT

In contrast to one-photon microscopy, two-photon probe-based fluorescence imaging can provide improved three-dimensional spatial localization and increased imaging depth. Therefore, the development of new functional two-photon fluorescent dye has attracted great attention. Herein, we have adopted the fluorescence resonance energy transfer (FRET) strategy to design a unique type of two-photon ratiometric fluorescent probe platform. Specifically, a two-photon fluorophore (D-π-A-structured naphthalimide derivative) and a rhodamine B fluorophore are directly connected by a flexible piperidine linker. We further used this platform to develop a new type of two-photon ratiometric fluorescent probe, **NRLys**, for lysosomal pH detection and bioimaging. The experiments demonstrated that the **NRLys** is a reliable and specific probe for labeling lysosomes in living cells and tissues with two well-resolved emission peaks separated by 60 nm. The probe showed high ratiometric imaging resolution and deep-tissue imaging depth of over 180 μm. Based on the above results, we expect that the new platform may prompt the development of a wide variety of two-photon ratiometric fluorescent probes application in biological systems.

1. Introduction

Fluorescent imaging is a widely used technology to investigate biological events with high spatiotemporal resolution. However, in the past, most of the fluorescent probes are one-photon fluorescent probes, they are excited with one-photon at short wavelengths, which causes a series of problems including photobleaching, interference from auto-fluorescence in cells and tissues, and low penetration depth (<80 μm). In contrast to one-photon microscopy, two-photon fluorescence imaging can overcome most of the aforementioned shortcomings, and provide improved three-dimensional spatial localization and increased imaging depth [1–3]. Most of the previously reported two-photon probes were designed based on the change of single-emission intensity changes, which could be affected by instrumental efficiency, environmental conditions, and the concentration of probe molecules [4–7]. However, the development of ratiometric probes eliminated most, if not all of, such interferences by a built-in correction of the two emission peaks. In the past, several strategies have been adopted to design ratiometric probes, e.g. internal charge transfer and fluorescence resonance energy transfer (**FRET**). For ratiometric probes based on **FRET**, the donor is linked to the acceptor with a flexible linking group. As a result, such probes showed high energy transfer efficiency, and two well-resolved emission peaks with high imaging resolution. Herein, we have established a new two-photon ratiometric fluorescent probe platform by directly connecting a 1,8-naphthalimide derivative (large two-photon cross section) [8,9] with a rhodamine **B** fluorophore via **FRET** strategy. In our study, we developed a two-photon ratiometric fluorescent probe **NRLys** to investigate the pH change in lysosome, which is an important suborganelle in living cells. Lysosome contains numerous enzymes and proteins, and exhibits a variety of activities and functions at pH 4.5–5.5 [10]. In order to maintain the lysosomal pH, the protons are pumped into lysosomal interior by a membrane-bound ATPase. Importantly, the cellular apoptosis process is associated with the gradient decay of lysosomal proton which leads to the increase of the lysosomal pH [11–13]. The dysfunction of lysosomes would induce various pathologies including neurodegenerative diseases

* Corresponding author.
E-mail address: zhouly0817@163.com (L. Zhou).

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[14], cancer [15] and Alzheimer's disease [16,17]. Therefore, it is important to monitor the lysosomal pH changes in living cells and tissues.

Traditionally, to image and track the lysosomes in vivo, two main classes of pH-sensitive fluorescent probes have been developed. The first category is turn-on probes [18–21] (Rlyso [18], RM [19], and FR-Lysome [21]). These probes are featured with a ring-closed spirolactam unit, which could show H^+-triggered turn-on fluorescence signals. The second category is ratiometric probes based on spectral shift in the emission during proton binding [22]. However, these two categories of probes for cells and tissues imaging with significant shortcomings, they are based on one-photon excitation and single-emission intensity changes, or single fluorophore spectral shift. In our approach, the two-photon ratiometric fluorescent probe **NRLys** exhibits a selective and sensitive response toward pH ranging from 3.0 to 7.0 and a large spectral shift between the two emissions (60 nm, from 525 to 585 nm), allowing for the high-resolution and sensitive ratiometric detection of the targets. **NRLys** was then applied for dual-channel imaging of lysosomes in live cells and intact tissues with a thickness over 180 μm, and both achieved satisfactory results.

2. Experimental

2.1. Materials and instruments

Unless otherwise specified, all chemicals were obtained from commercial suppliers and used without further purification. Thin layer chromatography (TLC) was carried out using silica gel 60 F254, and column chromatography was conducted over silica gel (100–200 mesh). Both of them were obtained from Qingdao Ocean Chemicals (Qingdao, China). In all experiments, water used was doubly distilled and purified by a Milli-Q system (Millipore, USA). LC–MS analyses were performed using an Agilent 1100 HPLC/MSD spectrometer. Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). ^{1}HNMR and ^{13}CNMR spectra were obtained using a Bruker DRX-400 spectrometer with TMS as an internal standard. All chemical shifts are reported in the standard δ notation of parts per million. UV–vis absorption spectra were recorded in 1.0 cm path length quartz cuvettes on a Shimadzu 2450 UV–vis Spectrometer. Fluorescence images of HeLa cells were obtained using Olympus FV1000-MPE multiphoton laser scanning confocal microscope (Japan). Fluorescence measurements were carried out on a F4500 fluorescence spectrometer with excitation and emission slits set at 5.0 nm and 5.0 nm, respectively. The pH was measured with a Mettler-Toledo Delta 320 pH meter.

2.2. Spectrophotometric measurements

The fluorescence measurement experiments were measured in phosphate buffer solution (10 mM) with DMSO as co-solvent solution (H_2O/DMSO = 99:1, v/v). The pH value of PBS solution used was from 3. 0–7.0, which was achieved by adding minimal volumes of HCl solution or NaOH solution. The fluorescence emission spectra were recorded at excitation wavelength of 400 nm with emission wavelength ranging from 475 to 650 nm. A 1×10^{-3} M stock solution of probe was prepared by dissolving probe compound in DMSO. Procedure of calibration measurements with probe in the buffer with different pH followed: 20 μL stock solution of probe and 1980 μL PBS buffer solution with different pH were combined to afford a test solution, which contained 1×10^{-6}M of probe. The solutions of various testing species were prepared from NaCl, $CaCl_2$, $MgSO_2$, $CuCl_2{\cdot}H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$, using twice-distilled water with final concentrations of 0.0125 M, as well as, glutathione (GSH), cysteine (Cys), and glutamate (Glu) using twice-distilled water with final concentrations of 0.025 M. Procedure of selectivity experiments is as follows: for cations or anions, 20 μL stock solution of probe, 1948 μL PBS solution (pH 7.0) and 32 μL solution of each cation or anion were combined to afford a test solution, which contained 1×10^{-6} M of probe and 200 μM cation or anion; for amino acids, 20 μL stock solution of probe, 1900 μL PBS buffer solution (pH 7.0) and 80 μL solution of each amino acid were combined to afford a test solution, which contained 1×10^{-6} M of probe and 1 mM amino acid.

2.3. The fluorescent quantum yields and two-photon excited fluorescence measurement

The quantum yields of **NRLys** was calculated by comparison with rhodamine 6 G (ΦR = 0.95 in ethanol) as a reference using the following equation:

$$\Phi_F = IA_R(n/n_R)^2\Phi_F/I_RA \quad (1)$$

where Φ_F is the quantum yield, I is the integrated area under the fluorescence spectra, A is the absorbance, n is the refractive index of the solvent, and R refers to the reference rhodamine 6G.

The two-photon excited fluorescence was measured by using a Ti: sapphire femtosecond oscillator (SpectraPhysics Mai Tai) as the excitation source. The output laser pulses have a tunable central wavelength from 690 nm to 1020 nm with pulse duration of less than 100 fs and a repetition rate of 80.5 MHz. The laser beam was focused onto the samples using a lens with a focus length of 3.0 cm. The emission was collected at an angle of 90° to the direction of the excitation beam to minimize the scattering. The emission signal was directed into a CCD (Princeton Instruments, Pixis 400B) coupled monochromator (IsoPlane160) with an optical fiber. A 750 nm short pass filter was placed before the spectrometer to minimize the scattering from the excitation light. The two photon absorption (TPA) cross section (δ) of the sample (s) at each wavelength was calculated according to Eq. (2), and rhodamine B in CH_3OH was used as the reference (r).

$$\delta = \delta_r(S_s\Phi_r\phi_rc_r)/(S_r\Phi_s\phi_sc_s) \quad (2)$$

where S is the integrated fluorescence intensity, Φ is the fluorescence quantum yield, C is the concentration of sample (s) and reference (r), and ϕ is the collection efficiency of the experimental setup. The uncertainty in the measurement of cross sections is ~15%. The detailed calculation is given in Fig. 2.

2.4. Cell cytotoxic assays and imaging

The cytotoxic effects of probe were assessed using the MTT assay. Immediately before the experiments, the cells were washed with PBS buffered solution, followed by incubating with probe (1 μM) for 30 min (in PBS containing 1% DMSO) at 37 °C, They were then washed with PBS three times and imaged. Lyso-Tracher blue (1 μM, Invitrogen) was used for co-staining experiments. Fluorescence microscopy imaging of HeLa cells were observed under an Olympus FV1000 laser confocal microscope. The fluorescent excitation wavelength was fixed at 405 nm and 485 nm. The fluorescent emissions wavelength were recorded at (425–475) nm, (495–540) and (560–650)nm individually. One- or two-photon fluorescence images of HeLa cells were obtained using an Olympus FV1000-MPE multiphoton laser scanning confocal microscope (Japan). The two-photon excitation wavelength was fixed at 780 nm.

2.5. Preparation and staining of nude mice muscle tissue slice

The slices were cultured with 10 μM **NRLys** in an incubator at 37 °C for 1 h and then washed with PBS three times for 2PFM imag-