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Fluorescence detection of intracellular pH changes in the mitochondria-associated process of mitophagy using a hemicyanine-based fluorescent probe

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ABSTRACT

Intracellular pH behaves as a vital parameter in the physiological and pathological processes. Novel small molecule probes for precise and dynamic monitoring of pH fluctuations in cellular physiological processes are still highly required. Herein, we present a hemicyanine-based probe (**HcP—H**) detection of the pH changes during the intracellular process of mitochondria-associated autophagy. **HcP-H** exhibits highly reversible and ratiometric fluorescence detection of pH variation due to the deprotonation/protonation process, showing orange fluorescence (λ_{em} = 557 nm) in basic media (pH 8.0) and green fluorescence (λ_{em} = 530 nm) in acidic media (pH 6.2), respectively. Organelle localization experiment in HeLa cells demonstrates that this probe could selectively accumulate in mitochondria, showing almost overlap with that of Mito-Tracker Green FM. More importantly, Fluorescence imaging of **HcP-H** in HeLa cells subjected to the nutrient deprivation has demonstrated that this probe could monitor the intracellular pH changes in the mitochondria-associated process of mitophagy. It is clearly confirmed that **HcP-H** would serve as a promising fluorescent probe for tracing mitophagy in living cells.

1. Introduction

Intracellular pH values behave as a vital parameter in cell proliferation, function and survival [1–3]. Under normal physiological conditions, subcellular organelles must hold individual pH levels in order to maintain respective characteristics in biological processes. For instance, the interior pH of lysosomes is in the range of 4.5–5.5, which steadily guarantee the optimum activity of hydrolytic enzymes in the degradation of redundant biomolecules such as proteins, nucleic acids, polysaccharides, lipids, and damaged organelles [4–8]. In contrast, the alkaline environment (approximately pH 8.0) is required for mitochondria to function properly. Slight changes of mitochondrial pH would lead to the excess production of reactive oxygen species (ROS) and result in damage to mitochondria, which are believed to be closely associated with cardiopulmonary and neurodegenerative diseases, as well as cancer and aging [9]. In addition, pH values in subcellular organelles dramatically fluctuate during cellular metabolic processes. For example, during mitochondria-associated autophagy, mitochondrial pH values gradually reduce from 8.0 to 4.5, indicating the membrane fusion process of alkaline mitochondria-containing autolysosomes with acidic lysosomes [10–12]. Therefore, in this regard, the pH values can serve as a key indicator associated with cellular functions and metabolic processes. And the development of strategies for the exact detection of variations in subcellular pH is highly desired for further understanding the physiological and pathological processes relevant to pH.

It is now generally accepted that fluorescent chemical probes have been recognized as a powerful analytical method owing to its high sensitivity, good selectivity and particularly, real-time and noninvasive detection in live cells or tissues [13–18]. Therefore, in recent years, numerous pH-responsive fluorescent probes have been reported with aiming at intracellular pH sensing and imaging. Among them, a few probes have successfully achieved monitoring pH fluctuation in homogeneous organelles including mitochondria, lysosomes, and endosomes.

In fact, the pH values not only vary inside individual organelles but also alter between different organelles, e. g. endocytosis and mitochondria-associated apoptosis. As for the latter physiological process, only a few fluorescent pH-responsive probes have been reported so far. Noteworthy are the works of Kim et al. who have reported a piperazine-linked naphthalimide off-on chemosensor monitoring of impaired mitochondria undergoing mitophagic elimination by virtue

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of its pH-sensitive in mitochondria [19]. Tan and colleagues who have developed a cyanine dye (HQO) for probing the mitophagy process in live cells based on its distinguishing pH-induced fluorescence (e.g. impaired mitochondria and autolysosomes) [20]. Han's group fabricated a hybridization of coumarin and spiro-lactam rhodamine framework, which used triphenylphosphine as the mitochondrial targeting group, to detect the autophagy relevant mitochondrial depolarization and lysosomal neutralization due to the distribution of probes inside different cellular organelles [21]. Most recently, Meng et al. developed a two-photo lysosome-targeting probe Lyso-OC for sensing autophagy by detection of the change in the lysosomal polarity during the process of autophagy [22]. While these fluorescent chemical probes abovementioned have achieved some success in detecting cellular physiological processes relevant to pH, there exist some disadvantages, such as selectivity, sensitivity, biocompatibility and organelle localization, which would disturb the precise and dynamic monitoring of pH fluctuations. Thus in this case, novel probes with specific characteristics in monitoring of pH fluctuations in mitophagy (autophagy) are still highly required.

Herein, we present a hemicyanine-based probe, **HcP-H** (4-[3-(1-Ethyl-3, 3-dimethyl-3H-indol-1-iodide-2-yl)-allyl]-phenol), for detection of the pH variation in mitochondria during the process of mitochondria-associated autophagy. The phenolic hydroxyl group is introduced to fulfill fluorescence alteration owing to the equilibrium between phenol and benzoquinone, which could change the conjugated system. Indole quaternary ammonium moiety, the excellent mitochondria targeting group as reported, is used as an anchor to selectively accumulate in mitochondria [23–28]. **HcP-H** showed strong orange fluorescence in neural/basic media, whereas the fluorescence emission became green when treated with an increasing gradient of acidity. Thus, we expected that such pH-sensitive emission of **HcP-H** would serve as a ratiometric fluorescent probe for tracing mitophagy in living cells.

2. Experimental

2.1. Materials and Instruments

All the reagents and solvents were purchased from commercial suppliers (J&K Scientific Ltd., China; Alfa Aesar, Thermo Fisher Scientific Ltd.). The solvents were of AR grade and were dried according to standard procedures. All reactions were monitored by thin-layer chromatography (TLC) with silica gel F254 (300–400 mesh). Flash column chromatography was performed using silica gel 60 (200–300 mesh). Doubly distilled water was used throughout the experiments.

UV–vis absorption spectra were recorded on Shimadzu UV-2350 Spectrophotometer. Fluorescence emission spectra were obtained using Varian Cary Eclipse Fluorescence spectrophotometer. ^{1}H NMR and ^{13}C NMR were measured on a Bruker AV400 nuclear magnetic resonance spectrometer. Fluorescence images were performed on Leica tcs sp8 confocallaser-scanning microscope.

2.2. Synthesis of Compound **2**

4-(2-Hydroxyethyl) phenol (0.7 g, 5.2 mmol) was dissolved in DMSO (6.9 mL) containing triethylamine (1.4 mL, 10 mmol). Then SO_3-Pyridine (4.5 g,28.5 mmol) complex in DMSO (5 mL) was slowly added dropwise. The reaction mixture was stirred for 1 h at room temperature. After quenching with cold water (50 mL), the mixture was continued to stir for 5 min at room temperature. Then the reaction mixture was extracted with CH_2Cl_2 (50 mL × 3) and the organic phase was washed with water and brine (25 mL × 2), dried over sodium sulphate anhydrous and evaporated to dryness. The resulting residue was purified by column chromatography on silica gel (Petroleum ether/EtOAc = 5: 1) to obtain compound **2** as yellow oil (295 mg, 46%). ^{1}H NMR (300 MHz, Chloroform *d*) δ 9.67 (t, J = 2.0 Hz, 1H), 7.03 (d, J = 8.0 Hz, 2H), 6.90–6.72 (m, 2H), 3.61 (d, J = 2.5 Hz, 2H).

2.3. Synthesis of Probe **HcP-H** and **Hcp-Q**

1-Ethyl-2,3,3-trimethyl-*3H*-indolium iodide **3** (301.2 mg,1.0 mmol) and compound **2** (136.1 mg, 1.0 mmol) was dissolved in anhydrous ethanol (10 mL). Then the mixture was refluxed for 10 h, after being cooled to room temperature, the reaction was added with water (10 mL) and acidified by hydrochloric acid (1 M), then the reaction mixture was extracted with CH_2Cl_2 (20 mL × 3). After removal of solvents, the residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH = 10:1) to afford **HcP-H** as a dark yellow powder (249.0 mg, yield: 57.5%).

HcP-H was deprotonated in sodium hydroxide solution (1 M), and the reaction mixture was then extracted with CH_2Cl_2 (20 mL). After removal of solvents, the residue was purified by column chromatography on basic alumina (CH_2Cl_2/MeOH = 10:1) to afford HcP-Q as a pink powder (yield: 86.3%).

HcP—H: ^{1}H NMR (300 MHz, DMSO d_6) δ 11.08 (s, 1H), 8.41 (d, J = 16.0 Hz, 1H), 8.15 (d, J = 8.3 Hz, 2H), 7.87 (d, J = 7.2 Hz, 2H), 7.59 (t, J = 5.7 Hz, 2H), 7.47 (d, J = 16.2 Hz, 1H), 6.97 (d, J = 8.3 Hz, 2H), 4.65 (d, J = 7.7 Hz, 2H), 1.78 (s, 6H), 1.43 (t, J = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, DMSO d_6) δ 180.93, 163.59, 157.99, 157.58, 154.69, 143.47, 140.41, 133.78, 128.96, 128.72, 125.87, 122.94, 119.22, 116.44, 114.47, 108.41, 51.76, 25.85, 13.44. HRMS (ESI) m/z calcd. For $C_{21}H_{24}NO^+$ $(M)^+$ 306.18524, found 306.18539.

HcP-Q: ^{1}H NMR (300 MHz, DMSO d_6) δ 8.17 (d, J = 15.2 Hz, 1H), 7.98 (d, J = 8.7 Hz, 2H), 7.72 (d, J = 7.5 Hz, 1H), 7.61 (d, J = 8.1 Hz, 1H), 7.50 (s, 1H), 7.41 (d, J = 7.4 Hz, 1H), 7.06 (d, J = 15.4 Hz, 2H), 6.66 (d, J = 8.7 Hz, 3H), 4.44 (d, J = 7.6 Hz, 2H), 2.50 (d, J = 3.2 Hz, 6H), 1.72 (s, 6H), 1.35 (s, 2H). ^{13}C NMR (75 MHz, DMSO d_6) δ 177.76, 158.03, 157.61, 152.68, 142.68, 140.93, 134.72, 128.82, 127.26, 124.42, 122.82, 119.27, 118.74, 113.17, 104.77, 60.38, 50.73, 26.37, 13.09. HRMS (ESI) m/z calcd. for $C_{21}H_{22}NO^+$ $(M)^+$ 304.16971, found 304.16971.

2.4. Spectral Measurements

A stock solution of probe (1.0 mM) was prepared in DMSO. Disodium hydrogen phosphate-citric acid buffer solutions consist of disodium hydrogen phosphate (0.2 M) and citric acid (0.1 M) and the gradient of pH values were achieved by changing the volume ratio of the two solutions. Test solutions were prepared by adding 40 μL of the probe stock solution into a quartz optical cell with 1.0 cm optical path length, and diluted with corresponding disodium hydrogen phosphate-citric acid buffer solutions to 4.0 mL. For selectivity experiments, all the stock solutions of cations and anions (i.e. K^+, Mg^{2+}, Cu^{2+}, Co^{2+}, Pb^{2+}, Fe^{3+}, Zn^{2+}, Ag^+, Cd^{2+}, Sn^{2+}, Mn^{2+}, Al^{3+}, HSO_3^-, $S_2O_3^{2-}$, HS^-) were prepared from the corresponding hydrochloride salts or sodium salts in double distilled water at a concentration of 10 mM, Test solutions were prepared by placing 40 μL of the probe stock solution and 40 μL of the solutions of ions into a quartz optical cell with 1.0 cm optical path length, diluting the solution to 4.0 mL with disodium hydrogen phosphate-citric acid buffer solutions at pH = 6.0 and pH = 8.0, respectively. Absorption and emission spectra were obtained on UV–vis and fluorescence spectrophotometer, respectively.

2.5. Cell Culture and Fluorescence Imaging

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma) with 10% newborn calf serum, as well as 5 mM L-glutamine and 5 mg/mL gentamicin. When the cells with stable growth in culture reached 85%–95%, cells were seeded in 35-mm glass-bottom culture dishes (Φ20 mm) and allowed to adhere for 48 h. For fluorescence imaging experiments, cells were washed three times