A Benzimidazole-based Ratiometric Fluorescent Probe for Accurate and Rapid Monitoring of Lysosomal pH in Cell Autophagy and Anticounterfeiting

Chaochao Wen, Jinyin Ge,* Yu Huang, Tao Gong, Congying Wang, Baofeng Yu* and Wenting Liang*

a Institute of Environmental Science, Department of Chemistry, College of Environmental and Resource Sciences, Shanxi University, Taiyuan 030006, China. E-mail: liangwt@sxu.edu.cn (W.L.);

b Department of Biochemistry and Molecular Biology, Shanxi Medical University, Taiyuan 030001, China. E-mail: shanxiyangcheng@126.com (B.Y.)

c Center for AIE Research, Shenzhen Key Laboratory of Polymer Science and Technology, Guangdong Research Center for Interfacial Engineering of Functional Materials, College of Materials Science and Engineering, Shenzhen University, Shenzhen 518060, China. E-mail: jyge0604@163.com (J.G.)

d Shaanxi Key Laboratory of Land Consolidation, School of Earth Science and Resources, Chang'an University, Xi'an 710064, China

1. Synthesis Steps and Characterization of Probe BD, OD and ID

Fig. S1 Schematic of the synthesis of probes BD, OD and ID

P-Dimethylaminobenzaldehyde and 2-methylbenzimidazole were mixed in N, N-dimethylformamide. After adding an appropriate amount of trimethylchlorosilane, the mixture was sealed and stirred at 110 °C for 24 hours. When cooling to room temperature, add an aqueous solution of saturated sodium carbonate to the mixture, adjust the pH to alkaline, and continue stirring for 30 minutes. Then, the reaction product was extracted with dichloromethane and saturated sodium chloride aqueous solution, the organic phase was collected, and the solvent was removed by evaporation under reduced pressure. Finally, the crude product was purified by silica
gel column chromatography (eluent PE/EA = 3/1, v/v) to obtain yellow powder BD. BD was characterized by 1H NMR, 13C NMR and MS (MALDI TOF), as Figure S2, S3 and S4. 1H NMR (600 MHz, DMSO) δ 12.44 (s, 1H), 7.56 (s, 1H), 7.53 (s, 1H), 7.49 (s, 1H), 7.48 (s, 1H), 7.13 (d, J = 3.6 Hz, 2H), 6.94 (s, 1H), 6.91 (s, 1H), 6.76 (s, 1H), 6.74 (s, 1H), 2.96 (s, 6H). 13C NMR (101 MHz, DMSO) δ: 152.37 – 152.34 (m), 151.35 – 151.06 (m), 135.42 – 135.37 (m), 128.74 – 128.72 (m), 123.82 – 123.80 (m), 122.13 – 122.10 (m), 112.69 – 112.68 (m), 112.62 – 112.61 (m), 40.30 – 40.30 (m). MS (MALDI TOF) m/z 264.1501 for [M+H]+.

Probe OD and ID were synthesized similarly to BD except that 2-methylbenzimidazole was exchanged for an equal amount of 2-methylbenzoxazole or 2,3,3-trimethylindole.

OD was characterized by 1H NMR, 13C NMR, as Figure S5, S6. 1H NMR (400 MHz, DMSO) δ 7.72 (s, 1H), 7.68 (s, 1H), 7.62 (s, 1H), 7.60 (s, 1H), 7.35 (d, J = 1.6 Hz, 1H), 7.33 (s, 1H), 6.98 (s, 1H), 6.94 (s, 1H), 6.76 (s, 1H), 6.74 (s, 1H), 2.99 (s, 6H). 13C NMR (101 MHz, DMSO) δ 163.95 – 163.91 (m), 151.91 – 151.88 (m), 150.23 – 150.16 (m), 142.50 – 142.46 (m), 140.62 – 140.61 (m), 129.89 – 129.88 (m), 125.19 – 125.17 (m), 124.94 – 124.93 (m), 122.78 – 122.75 (m), 119.48 – 119.47 (m), 112.37 – 112.35 (m), 110.70 – 110.69 (m), 108.15 – 108.14 (m), 40.19 – 40.18 (m).

ID was characterized by 1H NMR, 13C NMR, as Figure S7, S8. 1H NMR (400 MHz, DMSO) δ 7.62 (dd, J = 19.3, 12.5 Hz, 3H), 7.44 (dd, J = 12.7, 7.4 Hz, 2H), 7.28 (td, J = 7.6, 1.1 Hz, 1H), 7.17 (td, J = 7.4, 0.8 Hz, 1H), 6.96 (d, J = 16.2 Hz, 1H), 6.75 (d, J = 8.8 Hz, 2H), 2.98 (s, 6H), 1.37 (d, J = 12.3 Hz, 6H). 13C NMR (101 MHz, DMSO) δ 184.11 – 184.04 (m), 154.57 – 154.47 (m), 151.63 – 151.51 (m), 147.06 – 146.99 (m), 138.68 – 138.66 (m), 129.65 – 129.63 (m), 127.97 – 127.95 (m), 125.15 – 125.13 (m), 123.95 – 123.87 (m), 121.84 – 121.83 (m), 119.87 – 119.85 (m), 114.80 – 114.78 (m), 112.42 – 112.40 (m), 52.49 – 52.47 (m), 40.25 – 40.24 (m), 23.77 – 23.75 (m).

2. MTT Assay Procedure

SNU-423 cells in the exponential growth phase were seeded in a 96-well microtiter plate at a density of 5000/well, and incubated in a 37 °C incubator with a carbon dioxide concentration of 5%. After 24 hours, the BD solution with the concentration of 0 (control), 1, 10, 50, 100, 200 μM was prepared with fresh medium, and added to the microwells of different groups at 200 μL per
well to replace the original medium. After continued incubation for another 24 hours, cells were gently washed three times with phosphate buffered saline (PBS) and then injected into each well with 20μL MTT working solution (10 mg/mL in PBS). After 4 hours, the MTT solution in each well was discarded, and the cells were treated with 150 μL DMSO to dissolve the intracellular formazan crystals. The absorbance value of each well solution at the wavelength of 490 nm was determined using a microplate reader at room temperature. Cell viability was expressed by the percentage of OD values in the study group relative to the control group.

3. Supplementary Figures

Fig. S2 ¹H NMR spectrum of BD
Fig. S3 $^{13}$C NMR spectrum of BD

Fig. S4 HR-MS spectrum of BD
Fig. S5 $^1$H NMR spectrum of OD

Fig. S6 $^{13}$C NMR spectrum of OD
Fig. S7 $^1$H NMR spectrum of ID

Fig. S8 $^{13}$C NMR spectrum of ID
**Fig. S9** The optical performance of OD. (a) The absorption spectra of OD (10 μM) in a solution of pH 7.0-1.6. (b) The fluorescence spectra of OD (10 μM) in a solution of pH 7.0-1.6, $\lambda_{ex} = 350$ nm. (c) The pH (7.0-1.6) dependent Raito ($F_{538\text{nm}}/F_{405\text{nm}}$) sigmoidal fitting graph. Error bars represent standard deviation, n=5.
Fig. S10 The optical performance of ID. (a) The absorption spectra of ID (10 μM) in a solution of pH 9.5-2.6. (b) The fluorescence spectra of ID (10 μM) in a solution of pH 9.5-2.6, $\lambda_{ex} = 450$ nm. (c) The pH (9.5-2.6) dependent fluorescence intensity (553 nm) sigmoidal fitting graph. Error bars represent standard deviation, n=5.

Fig. S11 Predicted emission wavelength compared to measured emission wavelength. (a) Before protonation. (b) After protonation.
Fig. S12 (a) Raito (F_{487nm}/F_{534nm}) of BD (10 μM) in the presence of several cations, anions, and biomolecular interferences at pH 2.5, 4.7, and 7.0. 1, blank; 2, Na⁺ (10 mM); 3, K⁺ (10 mM); 4, Mg²⁺ (10 mM); 5, Cd²⁺ (0.5 mM); 6, Ca²⁺ (0.5 mM); 7, Co²⁺ (0.5 mM); 8, Ba²⁺ (0.5 mM); 9, Cu²⁺ (0.5 mM); 10, Zn²⁺ (0.5 mM); 11, Fe³⁺ (0.5 mM); 12, Al³⁺ (0.5 mM); 13, NH₄⁺ (0.5 mM); 14, chloroquine (0.5 mM); 15, hydrogen peroxide (0.5 mM); 16, N-acetylcysteine (0.5 mM); 17, glutamate (0.5 mM); 18, leucine (0.5 mM); 19, valine (0.5 mM); 20, serine (0.5 mM); 21, cysteine (0.5 mM); 22, aspartate (0.5 mM); 23, phenylalanine (0.5 mM); 24, vitamin C (0.5 mM); 25, Cl⁻ (100 mM); 26, SO₄²⁻ (10 mM); 27, NO₃⁻ (0.5 mM); 28, PO₄³⁻ (0.5 mM); 29, CO₃²⁻ (0.5 mM); 30, C₂H₅O₂⁻ (0.5 mM). (b) The photostability of BD (10 μM) in pH 2.5, 4.7, and 7.0 solutions within 2 hours. (c) The pH reversibility of BD (pH=2.5 and 7.0). (d) Cytotoxicity of different concentrations of BD to SNU-423 cells. 1, Control; 2, 1 μM; 3, 10 μM; 4, 50 μM; 5, 100 μM; 6, 200 μM. Error bars represent standard deviation, n=5 (a-c) or n=6 (d).
Fig. S13 (a) Confocal images of probe **BD** on SNU-423 and HL-7702 cells. $\lambda_{ex} = 408$ nm, the blue channel $\lambda_{em} = 460$-500 nm, the green channel $\lambda_{em} = 510$-560 nm. (b) The mean fluorescence intensities of the blue and green channels and the corresponding ratiometric fluorescence values. Error bars represent standard deviation, n=5.