RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Journal Name

ARTICLE

A ratiometric lysosomal pH probe based on the imidazo[1,5-α]pyridine–rhodamine FRET and ICT system

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Guang-Jie Song[a,b,#], Su-Yun Bai[c,d,#], Xi Dai[a], Xiao-Qun Cao[b,*], Bao-Xiang Zhao[a,*]

A new pH-activatable ratiometric fluorescent probe (RhMP) has been developed, in which an imidazo[1,5-α]pyridine fluorophore as a fluorescence resonance energy transfer (FRET) donor linked to rhodamine B fluorophore as a FRET acceptor. The simultaneous fluorescence intensity enhancement of imidazo[1,5-α]pyridine and rhodamine B moieties along with the decrease of pH value should result from the integration of ICT and FRET processes. It's the first time to report a ratiometric fluorescent probe based on FRET system using imidazo[1,5-α]pyridine derivative as a fluorophore donor. As pK_a = 4.96, the fluorescence intensity ratio ($I_{476.5}/I_{577.5}$) of the probe displayed excellent pH-dependent performance and responded linearly to minor pH changes in the range of 4.0-5.8. The probe exhibited excellent selectivity among different metal cations and brilliant reversibility. In addition, RhMP has low cytotoxicity and has been successfully applied in HeLa cells. The fluorescence microscopic images demonstrated this probe could image weak acid pH changes of lysosome in live cells.

Introduction

Hydrion plays a significant role in cell proliferation, apoptosis, enzymatic activity and tumor growth.[1-5] As an important indication of cellular health, minor variations of intracellular pH may cause cellular dysfunction and serious diseases, such as colorectal cancer,[6] breast cancer[7] and cystic fibrosis.[8] Lysosome with acidic environment (pH 4.0-6.0)[9] is identified as the first defence line of cells.[10] Many diseases, such as cancer, stroke and Alzheimer, could be caused when the pH in lysosome changes.[11, 12] Therefore, quantitative detection of pH in biological environments makes good sense in cellular analysis or diagnosis.

Many detection methods have been used to measure pH value, such as acid-base indicator titration and potentiometric titration.[13-15] Compared with those, fluorescent probe has many advantages including high selectivity and sensitivity, good spatiotemporal resolution, convenient operation and possibility of real time monitoring.[16-19] Most reported fluorescent pH probes depended on the measurement of fluorescence intensity changes in single-emission windows. However, the signal output tends to be affected by many exterior factors, such as temperature, solvent polarity, probe concentration and instrument sensitivity.[20-23] A ratiometric probe with two emission peaks can resolve above issues via the self-calibration of two emission bands.[24-26] Therefore, more precise detection of pH could be obtained by means of ratiometric probe even in complicated biological systems.[27, 28] Constructing an FRET system is a wonderful way to fabricate a ratiometric fluorescent probe, because FRET-based ratiometric probes with large Stoke shifts can reduce fluorescence detection error and self-quenching.[29-35] To date, the reported ratiometric pH probes based on FRET are still rare compared with the demand.

In this study, we designed and synthesized a ratiometric pH probe RhMP with high selectivity and sensitivity based on FRET. The probe was fabricated by integrating the imidazo[1,5-α]pyridine moiety as the FRET donor and the rhodamine moiety as the FRET acceptor. Rhodamine moiety as an FRET acceptor has been widely used because of its excellent biocompatibility and photophysical properties.[27-29, 36-42] What's more, the rhodamine moiety is non-fluorescent at basic and neutral pH condition due to its spirocyclic structure. In acidic solution, the spirocyclic form of the rhodamine moiety transformed into the ring-opening form, resulting in strong fluorescence. On the other hand, we had synthesized a imidazo[1,5-a]pyridine-based pH fluorescent probe with fast response, high selectivity and sensitivity.[43] However, this probe was a single-emission probe. And so far, imidazo[1,5-

[a] School of Chemical Engineering, Taishan Medical University, Tai'an 271000, PR China. Tel: 0086-538-6236195. Email address: xqcao@tsmc.edu.cn
[b] Institute of Organic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, PR China. Tel: 0086-531-88366425, Fax: 0086-531-88564464. Email address: bxzhao@sdu.edu.cn
[c] Institute of Developmental Biology, School of Life Science, Shandong University, Jinan 250100, P.R. China.
[d] Institute of Developmental Biology, School of Life Science, Taishan Medical University, Tai'an 271000, PR China.
†Electronic Supplementary Information (ESI) available: Synthesis of Donor, supplementary some fluorescent spectras, characterization of the compound: NMR, HRMS spectra. See DOI: 10.1039/x0xx00000x

α]pyridine fluorophore is not used as an FRET donor. Thus, we have been focusing on the design and synthesis of ratiometric fluorescent probe based on FRET applying imidazo[1,5-α]pyridine. Through our research, we know the emission spectrums of imidazo[1,5-α]pyridine and rhodamine are well separated. The probe RhMP has been successfully applied in fluorescence imaging in HeLa cells and could selectively stain lysosome.

Materials and methods

Materials

All reagents and solvents were purchased from commercial sources and used without further purification. The solutions of metal ions were prepared from nitrate salts which were dissolved in deionized water. Deionized water was used throughout the process of absorption and fluorescence determination. All samples were prepared at room temperature, shaken for 10 s and rested for 3 h before UV-vis and fluorescence determination. Britton-Robinson (B-R) buffer was prepared with 40 mM acetic, boric acid, and phosphoric acid. Dilute hydrochloric acid or sodium hydroxide was used for tuning pH values. LysoSensor™ Green DND-189 was used as lysosome tracker (Invitrogen, America).

Instruments

Thin-layer chromatography (TLC) involved silica gel 60 F_{254} plates (Merck KGaA) and column chromatography involved silica gel (mesh 200–300). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 300 spectrometer, using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard. Melting points were determined with an XD-4 digital micro-melting-point apparatus. HRMS spectra were obtained on a Q-TOF6510 spectrograph (Agilent). UV-vis spectra were measured by the use of a Hitachi U-4100 spectrophotometer. Fluorescence measurements were performed on a Perkin–Elmer LS-55 luminescence spectrophotometer. Quartz cuvettes with a 1 cm path length and 3 mL volume were used for all measurements. The pH was determined with a model PHS-3C pH meter. The single crystals were measured on a Bruker-AXS CCD single-crystal diffractometer with graphite-monochromated MoK radiation source (λ = 0.71073Å).

Preparation of probe RhMP

Ethyl 3-butyl-1-chloroimidazo[1,5-α]pyridine-7-carboxylate **1** can be easily synthesized by the reactions of commercially available 2-butyl-4-chloro-1H-imidazole-5-carbaldehyde and ethyl 4-bromobut-2-enoate in a tandem reaction way described previously in the literature.[44] Compound **1** (2.8 g, 10 mmol) and NaOH (0.8 g, 20 mmol) were dissolved in 20 mL ethanol and 15 mL water. The mixture was heated to reflux for 2 h. The reaction was monitored by TLC. The mixture was poured into water (200 mL) and acidified by hydrochloric acid (2 M). Yellow solid separated out and after filtration compound **2** was obtained in 92% yield. Compound **4** can be easily synthesized by the reaction of commercially rhodamine B and ethidene diamine described previously in the literature[44].

Compound **5** (RhMP) was obtained by the synthetic route (Scheme 1). Compound **2** (304.8 mg, 1.2 mmol), HOBT (162.0 mg, 1.2 mmol), EDC (229.2 mg, 1.2 mmol), Et_3N (202.0 mg, 2.0 mmol) were dissolved in 20 mL of DMF. Then the solution was stirred under N_2 for 1 h, and compound **4** was added to the solution. The mixture was stirred under N_2 atmosphere at room temperature for 20 h. The solvent was then evaporated under reduced pressure, and the crude product was dissolved in 150 mL CH_2Cl_2. The organic layer was washed with water (100 mL × 3), dried over anhydrous Na_2SO_4 and filtered. The combined organic phase was concentrated and then purified by column chromatography (silica gel, dichloromethane/methanol = 10:1, v:v) to give compound **5** in 41% yield. Faint yellow solid, Mp: 210-214 ℃. ^{1}H NMR (DMSO-d_6, 300 MHz), δ (ppm) = 8.43 (s, 1H), 8.19 (d, *J* = 7.5 Hz, 1H), 7.88 (s, 1H), 7.77-7.81 (m, 1H), 7.47-7.52 (m, 2H), 6.96-6.99 (m, 2H), 6.26-6.41 (m, 6H), 3.18-3.35 (m, 12H), 2.95 (d, *J* = 7.5 Hz, 2H), 1.69 (t, *J* = 7.5 Hz, 2H), 1.36 (q, *J* = 7.5 Hz, 2H), 1.03 (t, *J* = 7.5 Hz, 12H), 0.90 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (DMSO-d_6, 75 MHz), δ (ppm) = 12.29 (2C), 13.57 (2C), 21.66, 25.13, 28.56, 38.68, 43.54 (2C), 64.31, 97.30 (2C), 104.77 (2C), 108.08 (2C), 110.94 (2C), 116.02 (2C), 119.28, 121.45, 122.21, 123.54 (3C), 123.61 (2C), 128.24 (2C), 130.06, 132.64, 138.44 (2C), 148.21, 152.52 (3C), 153.87, 163.76, 167.70. HRMS ($C_{42}H_{47}ClN_6O_3$): Calcd. $[M+H]^+$: 719.3531; found value $[M+H]^+$: 719.3476.

Results and discussion

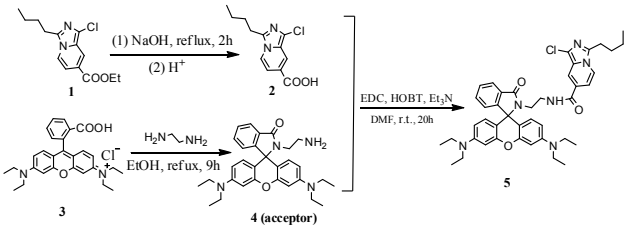
Design and synthesis of the probe

Probe RhMP, which was made from a imidazo[1,5-α]pyridine donor and a rhodamine acceptor, was designed as a ratiometric pH probe based on the FRET mechanism. The rhodamine derivative has been extensively used as a fluorophore by virtue of its excellent photophysical properties, such as long absorption and emission wavelength, high fluorescence quantum yield. The change in structure between spirocyclic (non-fluorescent) and ring-opening (fluorescent) forms in response to pH, in particular, was important. On the other hand, we had synthesized a imidazo[1,5-a]pyridine-based single-emission pH fluorescent probe.[43]

In this work, we have been focusing on the design and synthesis of ratiometric fluorescent probe based on FRET applying imidazo[1,5-α]pyridine. Imidazo[1,5-α]pyridine derivative is also a wonderful fluorophore with high fluorescence quantum yield and broad emission band. The broad band makes it possible to raise the spectral overlap between its emission spectrum and the absorption spectrum of the acceptor. The spectral overlap is beneficial to FRET mechanism. Therefore, rhodamine B moiety and imidazo[1,5-α]pyridine moiety are suited to FRET system. The synthetic route of probe RhMP was given in Scheme 1. The structure of the probe was characterized by ^{1}H NMR, ^{13}C NMR, HRMS spectra, especially, by X-ray single crystal diffraction (CCDC No. 1435699, Fig. 1).

Journal Name

ARTICLE



Scheme 1 The synthetic route of probe RhMP.

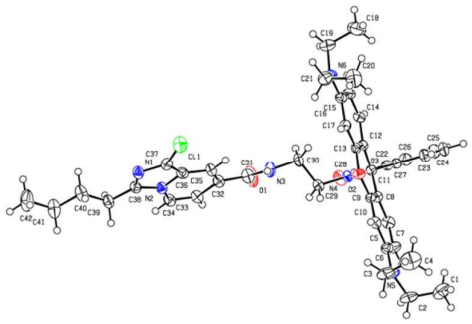


Fig. 1 The crystal structure of probe RhMP.

Spectroscopic properties

Fluorescence spectra and UV spectra properties of RhMP in the solution (B-R buffer/EtOH = 1/1, v/v, Fig. S1) were studied at diverse pH values (Fig. 2a and S2). RhMP exhibited relatively weak fluorescence at 476.5 nm and no emission peak centred at 577.5 nm under neutral conditions with an excitation wavelength of 355 nm. When the pH decreased from 7.2 to 4.0, the emission band of imidazo[1,5-α]pyridine centred at 476.5 nm enhanced gradually, at the same time, a remarkably fluorescence turn-on response at 577.5 nm was observed. The simultaneous intensity enhancement of imidazo[1,5-α]pyridine and rhodamine B along with the pH value decrease could be explained. Imidazo[1,5-α]pyridine as the FRET donor exerts a mechanism of ICT process.[30] In neutral condition, the nitrogen in position 2 can donate electron to carboxide group which linked to imidazo[1,5-α]pyridine to form a push-pull system. When 2-nitrogen is protonated, it is hard to donate electron to carboxide group because 2-nitrogen's electron density becomes poor. And this changes the intermolecular charge transfer (ICT). So the fluorescence intensity of imidazo[1,5-α]pyridine moiety changes with the pH changes. Meanwhile, the rhodamine B moiety is non-fluorescent at neutral pH condition due to its spirocyclic structure. In acidic solution, the spirocyclic form of the rhodamine moiety transformed into the ring-opening form, and the ring-opening form of rhodamine was an effective energy acceptor, the FRET process from the imidazo[1,5-α]pyridine donor to the rhodamine acceptor occurred accordingly, inducing strong fluorescence (Fig. S3-S6) Hence, the simultaneous intensity enhancement of imidazo[1,5-α]pyridine and rhodamine B along with the pH value decrease could be observed (Scheme 2). Moreover, energy transfer efficiency (*E*) was calculated to be 89.4 %, using the following way as reported.[45, 46] Quantum yield (Φ_F) was determined by the relative comparison procedure, with quinine sulfate dehydrate (Φ_{Fs} = 0.56, 0.1 N H_2SO_4) as the main standard. The general equation used in the determination of relative quantum yields from earlier research is as below :

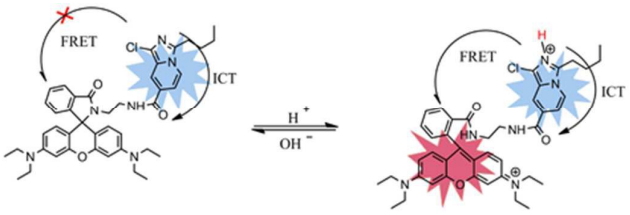
$\Phi_F = (\Phi_{Fs})(F_{Au})(A_s)(\eta^2_u) / (F_{As})(A_u)(\eta^2_s)$

Where Φ_F and F_A are fluorescence quantum yield and integrated area under the corrected emission spectrum, respectively; A is absorbance at the excitation wavelength; η is the refractive index of the solution; and subscripts u and s refer to the unknown and the standard, respectively. $\Phi_{F(\text{free donor})}$ = 0.217 (pH = 4.0); $\Phi_{F(\text{donor in FRET system})}$ = 0.023 (pH = 4.0).

Energy transfer efficiency (*E*) was calculated using the following equation as reported.

$E = 1 - \Phi_{F(\text{donor in FRET system})} / \Phi_{F(\text{free donor})} = 1 - 0.023/0.217 = 89.4\ \%$ (pH = 4.0).

When the pH value is between 4.0 and 5.8, the fluorescence intensity (Y axis) and the pH value (X axis) can be described by a perfect linear regression relationship with R^2 = 0.9973 (Fig. 2b). They can be described by the following formula pH = 3.25 + 0.18y ($y = I_{476.5}/I_{577.5}$). Any samples with pH ranged from 4.0 to 5.8 can be calculated by the formula based on their fluorescence intensity with high preciseness.



Scheme 2 The proposed ICT and FRET processes of probe RhMP toward pH.

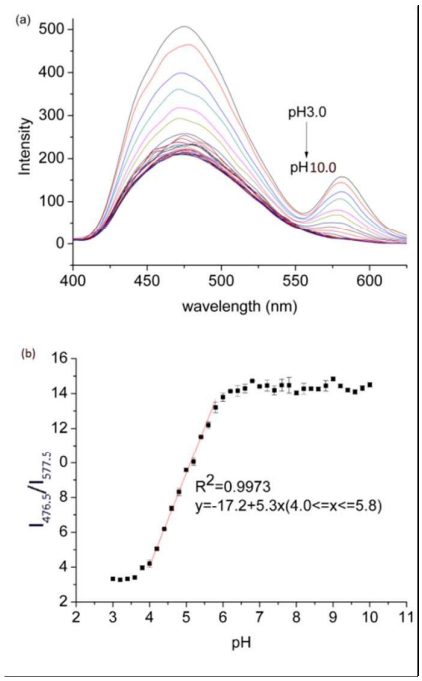


Fig. 2 (a) Fluorescence spectra of RhMP (10 μM) in the solution (B-R buffer/EtOH = 1/1, v/v) at different pH values (3.0-10.0); and (b) the plot of the emission ratios of RhMP at $I_{476.5}/I_{577.5}$ versus pH values including the linear relationship of the emission ratios ($I_{476.5}/I_{577.5}$) and pH values from 4.0 to 5.8 (R^2 = 0.9973), (λ_{ex} = 355 nm, slit: 10 nm/5 nm).

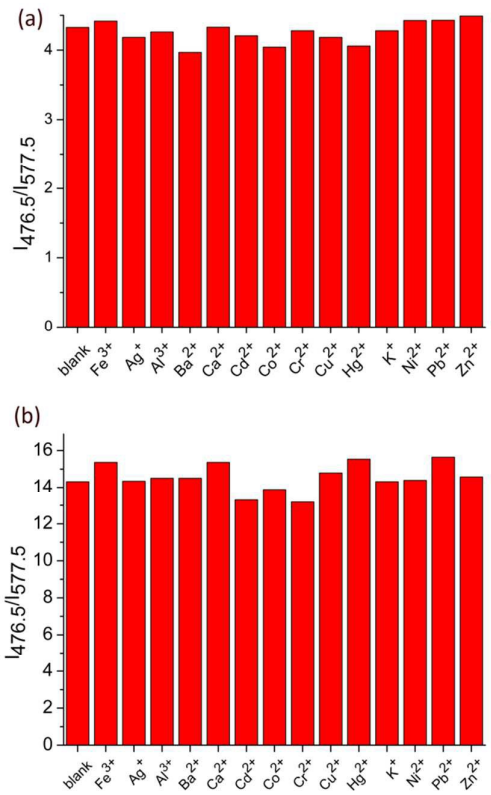


Fig. 3 The ratio of fluorescence intensity of RhMP (10 μM) at $I_{476.5}/I_{577.5}$ containing different metal cations in the solution (B-R buffer/EtOH = 1/1, v/v) at pH 4.0 (a) and pH 7.0 (b), All the concentration of the metal cations are 100 μM. (λ_{ex} = 355 nm, slit: 10 nm/5 nm).

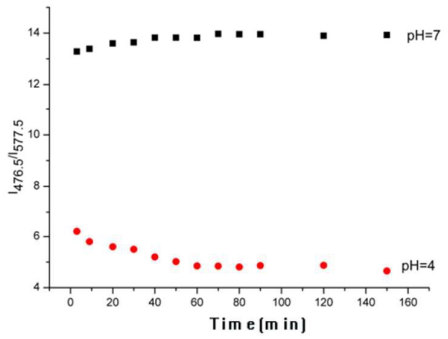


Fig. 4 Time courses of the fluorescence emission rations $I_{476.5}/I_{577.5}$ of RhMP (10 μM) in the solution (B-R buffer/EtOH = 1/1, v/v) at pH 4.0 and pH 7.0. (λ_{ex} = 355 nm, slit: 10 nm/5 nm).

The protonation of the 2-nitrogen in imidazole has pivotal affection to the probe as mentioned above. However, many metal ions may bind to nitrogen, so metal ions may have potential harmful to the probe. Therefore, it is necessary to detect if the metal ions could cause interference. The results demonstrated that the probe RhMP displayed admirable selectivity to H^+ over the above metal ions under pH = 4.0 and pH = 7.0 (Fig. 3, Fig. S7 and S8).

We detected the time courses of the fluorescence emission rations $I_{476.5}/I_{577.5}$ of RhMP (10 μM) in the solution (B-R buffer/EtOH = 1/1, v/v) at pH 4.0 and pH 7.0. The fluorescence emission rations $I_{476.5}/I_{577.5}$ of RhMP reached stable state in 50 min and remained stable for a long time at room temperature (Fig. 4).

Furthermore, we also investigated pH reversibility of RhMP (10 μM) in the solution (B-R buffer /EtOH = 1/1, v/v) at pH 4.0 and pH 7.0 (Fig. S9). RhMP revealed good pH reversibility.

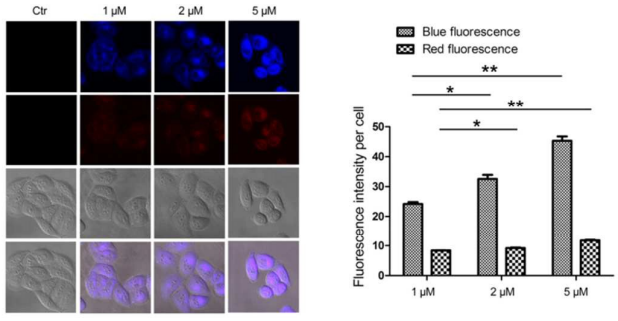


Fig. 5 Fluorescence microscope images of living HeLa cells incubated with the probe (1, 2 or 5 μM) for 2 h at 37 ℃. (a) Ctr: without the probe. First line:

fluorescence images at the blue channel (400–555 nm), second line: fluorescence images at the red channel (560–700 nm), third line: bright field images, fourth line: overlay images of the first, second and third lines. λ_{ex} = 405 nm. (b) fluorescence intensity quantitation was analyzed using the Image J. The results were presented as mean ± SE (*p < 0.05 and ** p < 0.01; n = 3).

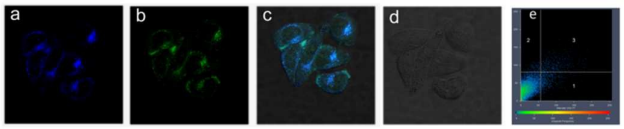


Fig. 6 The probe co-localized with lysosomes in living HeLa cells. (a): probe (2 μM)，(b): LysoSensors Green DND-189 (0.5 mM), (c): overlay of (a) and (b), (d): a bright field image, (e): Quantitation of co-localization coefficients for RhMP and LysoSensors Green DND-189. Excitation wavelength of RhMP and LysoSensor Green DND-189 were 405 nm and 488 nm, respectively; Emission channel for RhMP and LysoSensor Green DND-189 were 400-555 nm and 488-700 nm, respectively.

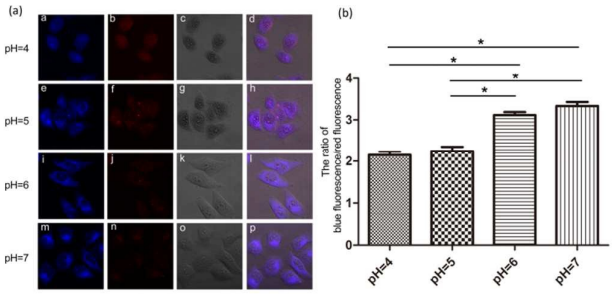


Fig. 7 (a) Fluorescence microscopy images of HeLa cells incubated with indicated pH PBS buffers containing nigericin (10 μM) for 0.5 h after treatment with the probe (2 μM) for 2 h. The pH of PBS buffer is 4.0 (a-d), pH 5.0 (e-h), pH 6.0 (i-l), and pH 7.0 (m–p), respectively. First column: fluorescence images at the blue channel (400–555 nm), second column: fluorescence images at the red channel (560–700 nm), third column: bright field images, fourth column: overlay images of first, second and third column. λ_{ex} = 405 nm (b) fluorescence intensity quantitation was analyzed using the Image J. The results were presented as mean±SE (*p < 0.05 and **p < 0.01; n = 3).

Fluorescence imaging in cell

Then, we applied probe RhMP to living cell imaging assays to investigate whether the probe could detect H^+ sensitively in biological systems. HeLa cells were incubated with the probe (1, 2 or 5 μM) for 2 h at 37 ℃. Both blue and red fluorescence intensities in HeLa cells increased along with the increase of concentration of the probe, indicating that probe RhMP possessed the ability of staining in living cells (Fig. 5). To further explore the distribution of RhMP within the cells, the Lysosensor™ Green DND-189 as an excellent lysosome tracker was used to stain HeLa cells with probe RhMP. HeLa cells were incubated with probe RhMP with (10 μM) for 2 h at 37 ℃, and the fresh medium containing LysoSensor® Green DND-189 (1 μM) replaced the former medium and further incubated for 1 h. The results demonstrated that probe RhMP could selectively stain lysosome in HeLa cells and the co-localization coefficient is 0.904 (Fig. 6). Subsequently, HeLa cells were incubated with RhMP (2 μM) for 2 h in different pH values (4.00, 5.00, 6.00 and 7.00) respectively followed by incubation with indicated pH PBS buffers containing nigericin (10 μM) for 0.5 h. The fluorescence of RhMP within the cells was observed by confocal fluorescence microscopy. Blue fluorescence of the imidazo[1,5-a]pyridine moiety and red fluorescence of the rhodamine B moiety increased simultaneous along with pH decreased from 7.0 to 4.0 (Fig. 7 a) and the ratio of blue fluorescence/red fluorescence increased along with pH decreased from 4.0 to 7.0 (Fig. 7 b). This result coincided with the result obtained in the B-R buffer solution test (Fig. 2). In addition, we also tested the photostability of the probe, as shown in Fig. S10, under a continuous excitation (3 min) using λ = 405 nm lasers, the ratio of the blue fluorescence /red fluorescence was essentially constant. This indicated that the photostability of the probe in HeLa cells is satisfactory. What's more, the effect of the probe on the viability of HeLa cells can be almost ignored (Fig. S11).

Conclusion

In summary, a new pH-activatable ratiometric fluorescent probe RhMP has been developed. RhMP was synthesized by integrating a imidazo[1,5-α]pyridine fluorophore as the fluorescence resonance energy transfer (FRET) donor and rhodamine B fluorophore as the FRET acceptor. The imidazo[1,5-α]pyridine fluorophore is the first time to be used as the fluorophore donor in the ratiometric fluorescent probe based on FRET. With a pK_a of 4.96, the fluorescence intensity ratio ($I_{476.5}/I_{577.5}$) of the probe displayed excellent pH-dependent performance and responded linearly to minor pH changes in the range of 4.0-5.8. The probe display admirable selectivity to H^+ among different metal cations and brilliant reversibility. In addition, RhMP has been successfully applied in HeLa cells, and the fluorescence microscopic images demonstrated this probe could electively stain lysosome in HeLa cells.

Acknowledgement

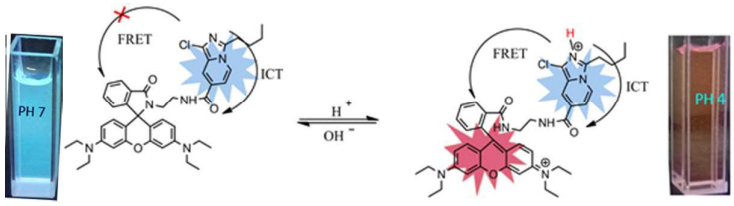
This study is supported by the Natural Science Foundation of Shandong Province (ZR2014BM004) and (ZR2013BM020).

Notes and references

1. R. Martinez Zaguilan, B. F. Chinnock, S. Wald-Hopkins, M. Bernas, D. Way, M. Weinand, M. H. Witte and R. J. Gillies, *Cell. Physiol. Biochem.*, 1996, **6**, 169.
2. S. Humez, M. Monet, F. Coppenolle, P. Delcourt and N. Prevarskaya, *Am. J. Physiol. Cell Physiol.*, 2004, **287**, 1733.
3. M. Czepan, Z. J. Rakonczay, A. Varro, I. Steele, R. Dimaline, N. Lertkowit, J. Lonovics, A. Schnur, G. Biczo, A. Geisz, G. Lazar, Z. Simonka, V. Venglovecz, T. Wittmann and P. Hegyi, *Pflugers Arch.*, 2012, **463**, 459.
4. J. K. Laihia, J. P. Kallio, P. Taimen, H. Kujari, V. M. Kahari and L. Leino, *J. Invest. Dermatol.*, 2010, **130**, 2431.
5. J. W. Wojtkowiak and R. J. Gillies, *Autophagy*, 2012, **8**, 1688.

6. A. Serafino, N. Moroni, R. Psaila, M. Zonfrillo, F. Andreola, F. Wannenes, L. Mercuri, G. Rasi and P. Pierimarchi, *Biochim. Biophys., Acta*, 2012, **1822**, 1004.
7. A. Rivinoja, N. Kokkonen, I. Kellokumpu and S. Kellokumpu, *J. Cell. Physiol.*, 2006, **208**, 167.
8. J. Poschet, E. Perkett and V. Deretic, *Trends Mol. Med*., 2002, **8**, 512.
9. H. Zhu, J. L. Fan, Q. L. Xu, H. L. Li, J. Y. Wang, P. Gao and X. J. Peng, *Chem. Commun.*, 2012, **48**, 11766.
10. J. L. Fan, C. Y. Lin, H. L. Li, P. Zhan, J. Y. Wang, S. Cui, M. M. Hua, G. H. Cheng and X. J. Peng, *Dyes Pigm.*, 2013, **99**, 620.
11. S. Q. Wu, Z. Li, J. H. Han and S. F. Han, *Chem. Commun*., 2011, **47**, 11276.
12. Z. Li, S. Q. Wu, J. H. Han and S. F. Han, *Analyst*, 2011, **136**, 3698.
13. D. Ellis and R. C. Thomas, *Nature*, 1976, **262**, 224.
14. R. G. Zhang, S. G. Kelen and J. C. LaManna, *J. Appl. Phys.*, 1990, **68**, 1101.
15. S. J. A. Hesse, G. J. G. Ruijter, C. Dijkema and J. Vissser, *J. Biotechnol.*, 2000, **77**, 5.
16. J. Y. Han, A. Loudet, R. Barhoumi, R. C. Burghardt and K. Burgess, *J. Am. Chem. Soc.*, 2009, **131**, 1642.
17. R. Huang, S. Y. Yan, X. L. Zheng, F. L. Luo, M. G. Deng, B. H. Fu, Y. J. Xiao, X. Zhao and X. Zhou, *Analyst*, 2012, **137**, 4418.
18. S. Xia, S. Y. Xiao, Q. Q. Hong, J. R. Zou, S. Yang, M. X. Zhang and H. Zuo, *RSC. Adv.*, 2015, **5**, 5244.
19. X. Dai, T. Zhang, Y. Z. Liu, T. Yan, Y. Li, J. Y. Miao and B. X. Zhao, *Sensor. Actuat. B-chem.*, 2015, **207**, 872.
20. H. S. Lv, J. Liu, J. Zhao, B. X. Zhao and J. Y. Miao, *Sensor. Actuat. B-chem.*, 2013, **177**, 956.
21. M. H. Lee, J. H. Han, J. H. Lee, N. Park, R. Kumar, C. Kang and J. S. Kim, *Angew. Chem. Int. Ed.*, 2013, **52**, 6206.
22. Y. H. Chan, C. F. Wu, F. M. Ye, Y. H. Jin, P. B. Smith and D. T. Chiu, *Anal. Chem.*, 2011, **83**, 1448.
23. H. S. Lv, S.Y. Huang, Y. Xu, X. Dai, J.Y. Miao and B. X. Zhao, *Bioorg. Med. Chem. lett*., 2014, **24**, 535.
24. M. H. Lee, J. H. Han, J. H. Lee, N. Park, R. Kumar, C. Kang and J. S. Kim, *Angew. Chem. Int. Ed.*, 2013, **52**, 6206.
25. X. F. Zhou, F. Y. Su, H. G. Lu, P. S. Willis, Y. Q. Tian, R. H. Johnson and D. R. Meldrum, *Biomaterials*, 2012, **33**, 171.
26. K. Kikuchi, H. Takakusa and T. Nagano, *TrAC, Trends Anal. Chem.*, 2004, **23**, 407
27. X. F. Zhang, T. Zhang, S.L. Shen, J.Y. Miao and B.X. Zhao, *RSC. Adv.*, 2015, **5**, 49115.
28. S. L. Shen, X. F. Zhang, S. Y. Bai, J. Y. Miao and B. X. Zhao, *RSC. Adv.*, 2015, **5**, 13341.
29. Y. R. Zhang, X. P. Chen, J. Shao. J. Y. Zhang, Q. Yuan, J. Y. Miao and B. X. Zhao, *Chem. Commun.* 2014, **91**, 14241.
30. K. Aich, S. Goswami, S. Das, C. D. Mukhopadhyay, K. Ching and H. K. Fun, *Inorg. Chem.*, 2015, **54**, 7309.
31. H. Yu, Y. Xiao, H. Guo and X. Qian, Chem. *Eur. J.,* 2011, **17**, 3179.
32. H. Yu, L. Jin, Y. Dai, H. Li and Y. Xiao, *New J. Chem.*, 2013, **37**, 1688.
33. S. Goswami, A. Manna, S. Paul, A. K. Maity, P. Saha, C. K. Quah, H. K. Fun, *RSC Adv.,* 2014. **4**. 34572.
34. S. Goswami, S. Paul, A. Manna, *Tetrahedron.,* 2014, **55**, 3946.
35. S. Goswami, S. Paul, A. Manna, *RSC Adv.,* 2014, **4**, 43778.
36. S. Goswami, S. Das, K. Aich, D. Sarkar, T. K. Mondal, C. K. Quah and H. K. Fun, *Dalton T.*, 2013, **42**, 15113.
37. S. Goswami, K. Aich, S. Das, A.K. Das, A. Manna and S. Halder, *Analyst*, 2013, **138**, 1903.
38. S. Goswami, S. Das, K. Aich, P. K. Nandi, K. Ghoshal, C. K. Quahc, M. Bhattacharyyab, H. K. Funcd and H. A. Abdel-Aziz, *RSC. Adv.*, 2014, **4**, 24881.
39. S. Goswami, A. Manna, S. Paul, *RSC Adv., 2014, **4**, 21984*
40. S. Paul, A. Manna, S. Goswami, *Dalton Trans.,* 2015, **44**, 11805.
41. S. Goswami, A. Manna, A. K. Maity, S. Paul, A. K. Das, M. K. Das, P. Saha, C. K. Quah, H. K. Fun, Dalton Trans., 2013, **42**, 12844.
42. S. Goswami, S. Paul, A. Manna, *RSC. Adv.,* 2013, **3**, 10639
43. X. Zhang, G. J. Song, X. J. Cao, J. T. Liu, M. Y. Chen, X. Q. Cao and B. X. Zhao, *RSC. Adv.*, 2015, **5**, 89827.
44. H. M. Li, C. Wang and J. W. Wang, *Chin. Chem. Lett.*, 2000, **11**, 949.
45. Y. l. Liu, X. Lv, Y. Zhao, M. l. Chen, J. Liu, P. Wang and W. Guo, *Dyes Pigments*, 2012, **92,** 909.
46. H. B. Yu, Y. Xiao, H. Y. Guo and X. H. Qian, *Chem. Eur. J.,* 2011, **17**, 3179.

A ratiometric lysosomal pH probe based on the imidazo[1,5-α]pyridine–rhodamine FRET and ICT system



A new pH-activatable ratiometric fluorescent probe (RhMP) has been developed based on FRET. This probe displayed good selectivity, excellent reversibility. In addition, RhMP has low cytotoxicity and has been successfully applied in HeLa cells.