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Ratiometric Near-Infrared Fluorescent Probes Based On Through-Bond Energy Transfer and π -Conjugation Modulation between Tetraphenylethene and Hemicyanine Moieties for Sensitive Detection of pH Changes in Live Cells

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Abstract

In this paper, we present three ratiometric near-infrared fluorescent probes (**A–C**) for accurate, ratiometric detection of intracellular pH changes in live cells. Probe **A** consists of a tetraphenylethene (TPE) donor and near-infrared hemicyanine acceptor in a through-bond energy transfer (TBET) strategy, while probes **B** and **C** are composed of TPE and hemicyanine moieties through single and double sp^2 carbon–carbon bond connections in a π -conjugation modulation strategy. The specific targeting of the probes to lysosomes in live cells was achieved by introducing morpholine residues to the hemicyanine moieties to form closed spirolactam ring structures. Probe **A** shows aggregation-induced emission (AIE) property at neutral or basic pH, while probes **B** and **C** lack AIE properties. At basic or neutral pH, the probes only show fluorescence of TPE moieties with closed spirolactam forms of hemicyanine moieties, and effectively avoid blind fluorescence imaging spots, an issue which typical intensity-based pH fluorescent probes encounter. Three probes show ratiometric fluorescence responses to pH changes from 7.0 to 3.0 with TPE fluorescence decreases and hemicyanine fluorescence increases, because acidic pH makes the spirolactam rings open to enhance π -conjugation of hemicyanine moieties. However, probe **A** shows much more sensitive ratiometric fluorescence responses to pH changes from 7.0 to 3.0 with remarkable ratio increase of TPE fluorescence to hemicyanine fluorescence up to 238-fold than probes **B** and **C** because of its high efficiency of energy transfer

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from TPE donor to the hemicyanine acceptor in the TBET strategy. The probe offers dual Stokes shifts with a large pseudo-Stokes shift of 361 nm and well-defined dual emissions, and allows for colocalization of the imaging readouts of visible and near-infrared fluorescence channels to achieve more precisely double-checked ratiometric fluorescence imaging. These platforms could be employed to develop a variety of novel ratiometric fluorescent probes for accurate detection of different analytes in applications of chemical and biological sensing, imaging, and diagnostics by introducing appropriate sensing ligands to hemicyanine moieties to form on–off spirolactam switches.

Graphical abstract

Introduction

Ratiometric fluorescence imaging shows better performance and reliability in quantitative and comparative analyses and effectively minimizes the interference of systematic errors such as the fluctuations of excitation light source, sample heterogeneity, uneven dye distribution, concentration variation, and compartmental localization of intensity-based fluorescent probes through a built-in calibration of dual emission bands.^{1–12} They have been commonly applied to study highly dynamic intracellular metal ions, biothiols, voltage, or pH changes in biological detection and imaging because of these comparatively ideal advantages.^{1–9,11–13} There are three different strategies to achieve ratiometric fluorescence imaging. The first strategy is to modulate π -conjugation of fluorophores in response to analytes.^{14–16} The second strategy is based on Forster resonance energy transfer (FRET) from a fluorophore donor to a fluorophore acceptor through space.^{7,11,12,17} This strategy lacks flexibility with limited choices of the donor and acceptor pairs because the donor emission must be substantially overlapped with the acceptor absorption in order to achieve effective FRET from the donor to the acceptor.^{11,17} In addition, FRET typically suffers from low energy transfer efficiency because of its energy transfer through space pathway, and often lacks dual well-defined fluorescence peaks.^{8,11,12,17} The third strategy employs through-bond energy transfer (TBET) from a fluorophore donor to a fluorophore acceptor where the donor and the acceptor are connected through a rigid electronic π -conjugation linker without a coplanar configuration, and the energy transfer is achieved through the electronically conjugated linker without the need for a substantial spectral overlap between

the donor emission and the acceptor absorption.^{7,10,11,18–20} This strategy offers great flexibility with more choices of the donor and acceptor pairs as long as these pairs have the same level of fluorescence quantum yields in order to achieve substantial ratiometric responses of the probes to analytes.^{10,18–21} Although a lot of fluorescent probes have been developed to detect pH,^{22–27} only a few ratiometric fluorescent probes were reported.^{7,28} Most of them are based on a FRET strategy with Rhodamine dyes with less than 600 nm in emission as acceptors.^{7,28} It is very challenging to develop near-infrared ratiometric fluorescent probes with well-defined dual emissions and excitations to achieve remarkable ratiometric fluorescence responses to pH because fluorescence quantum yields of infrared fluorescent dyes as acceptors in a FRET or TBET strategy are much lower than those with traditional Rhodamine acceptors. To the best of our knowledge, near-infrared ratiometric fluorescent probes have rarely been developed with near-infrared emission greater than 700 nm by using the TBET or FRET strategy. Therefore, it is essential to develop ratiometric near-infrared fluorescent probes with large dynamic responsive ranges, large pseudo-Stokes shifts with near-infrared emission, ideal ratio-metric fluorescence responses, photodamage-free imaging of living organisms, and low autofluorescence from biological samples for highly selective and sensitive ratiometric detection pH changes with well-defined dual excitation and emission features.

In this paper, we chose TPE as a donor because of its unique aggregation-induced emission (AIE) property,^{29–37} and employed TBET and π -conjugation modulation strategies to construct three ratiometric near-infrared fluorescent probes (**A**, **B**, and **C**) consisting of TPE and hemicyanine moieties, and investigated the effect of these strategies on probe ratiometric sensitivity to pH changes (Scheme 1). In order to achieve specific targeting to lysosomes in live cells, morpholine residue was chosen to attach to hemicyanine moieties to form closed spirolactam ring structures. Probe **A** is composed of TPE donor and hemicyanine acceptor based on a TBET strategy, while probes **B** and **C** consist of TPE and hemicyanine moieties through single and double sp^2 carbon–carbon connection based on a π -conjugation manipulation strategy. Probe **A** possesses AIE property under neutral and basic pH conditions while probes **B** and **C** lack AIE property. Three probes can provide reliable ratiometric responses to pH changes in both solution and live cells with TPE fluorescence decreases and hemicyanine fluorescence increases. Probe **A** exhibits much more sensitive ratiometric response to pH changes from 7.0 to 3.0 with significant ratio increase of TPE fluorescence to hemicyanine fluorescence up to 238-fold than probes **B** and **C** because of its highly efficient energy transfer from the TPE donor to the hemicyanine acceptor. Probe **A** offers well-defined visible and near-infrared fluorescence peaks with a large pseudo-Stokes shift of 361 nm, and provides excellent resolution to achieve extraordinary ratiometric fluorescence imaging of pH changes in live cells. The probes show low cytotoxicity, excellent photostability, good membrane permeability, excellent reversibility, and sensitive and selective ratiometric responses to pH over metal ions, anion ions, and amino acids.

Results and Discussion

Design and Synthetic Approach

We chose TPE as a donor in design of ratiometric probes because of its AIE properties.^{29–37} Although TPE has been used as donors of ratiometric probes in a FRET or TBET strategy, traditional Rhodamine dyes with less than 600 nm in emission were commonly used as acceptors, and ratiometric near-infrared fluorescent probes have not been receiving much attention. It is very challenging to achieve excellent ratiometric responses with well-defined dual emission peaks by using near-infrared fluorescent dyes as acceptors in a TBET or FRET strategy because of their much lower fluorescence quantum yields compared with those of traditional Rhodamine dyes.^{21,38–40} In this paper, we chose near-infrared hemicyanine dyes with both near-infrared absorption and emission as an acceptor in a TBET strategy because of their near-infrared fluorescence emission, high molar absorptivity, and excellent photostability with Rhodamine-like fluorescence on–off switching mechanism by spirocyclization.^{38,39,41} We designed three ratiometric fluorescent probes based on TBET and π -conjugation modulation strategies, and investigate the effect of different single and double sp^2 carbon–carbon bond connections between TPE and hemicyanine moieties on ratiometric fluorescence responses of the probes to pH changes in live cells (Scheme 2). In the TBET strategy, we prepared probe **A** by employing a palladium-catalyzed Suzuki reaction to electronically conjugate TPE donor (**8**) to hemicyanine acceptor (**7a**). The hemicyanine dye (**7a**) was prepared by coupling mixed hemicyanine dyes (**5a** and **5b**) with morpholine amine derivative (**8**) in the presence of BOP and triethylamine. In order to introduce TPE moiety to hemicyanine dye via single and double sp^2 carbon–carbon connections in the π -conjugation modulation strategy, we introduced an iodo group to hemicyanine dyes by condensation reaction of compound **11** with 5-iodo-2,3,3-trimethyl-1-(3-sulfopropyl)-3*H*-indolium inner salt (**12a**) and 5-iodo-1,2,3,3-tetramethyl-3*H*-indolium iodide (**12b**), affording iodo-functionalized hemicyanine dyes (**13a**, **13b**), respectively. In order to specifically target lysosome regions in live cells, morpholine residue was introduced to hemicyanine dyes **13a** and **13b**, affording hemicyanine dyes with closed spirolactam ring structures (**14a** and **14b**), respectively (Scheme 2). Ratiometric fluorescent probe bearing TPE and hemicyanine moieties via a single sp^2 carbon–carbon connection (**B**) was prepared by a palladium-catalyzed Suzuki coupling reaction of iodo-hemi-cyanine dye (**14a**) with 2-[4-[2,2-*bis*(4-methoxyphenyl)-1-phenylethenyl]phenyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**8**). The ratiometric fluorescent probe with TPE moiety connected to a hemicyanine moiety via a vinyl bond (**C**) was prepared by a palladium-catalyzed Heck coupling reaction of iodo-hemicyanine dye (**14b**) with 1-[2,2-*bis*(4-methoxyphenyl)-1-phenylethenyl]-4-ethenyl-benzene (**15**).

Study Aggregation-Induced Emission of the Probes

We employed TPE to develop ratiometric fluorescent probes because of its AIE property.^{29–37} We investigated whether TPE moieties of probes **A**–**C** maintain AIE property when they were conjugated to hemicyanine moieties. TPE absorbance of probes **A**, **B**, or **C** gradually decreases when water percentage in mixed ethanol and water solutions gradually increases from 0% to 90% (Figure S18 in Supporting Information). TPE moiety of probe **A** retains AIE property at neutral pH because the increase of water percentage in mixed ethanol

and water solutions from 0% to 70% results in significant fluorescence enhancement (Figure 1, left, and Figure 2). However, TPE moieties of probes **B** and **C** lack AIE properties as the water percentage increase in mixed ethanol and water solutions causes fluorescence quenching of TPE moieties of probes **B** and **C** (Figure 1, middle and right).

Absorption Responses of the Probes to pH Changes

Absorption responses of the probes to pH were investigated in 10 mM citrate buffers with different pH values containing 30% ethanol (Figure 3). Probe **A** exhibits an absorption peak of TPE donor at 376 nm under neutral and basic pH conditions. Probe **B** displays an absorption peak at 346 nm corresponding to the absorption peak of TPE moiety at a neutral or basic pH with closed spirolactam structure of the hemicyanine moiety while probe **C** shows the absorption peak of TPE moiety at 413 nm under the same pH condition. Probe **A** displays a new main near-infrared absorption peak at 715 nm and a shoulder deep-red absorption peak at 656 nm at pH 2.5, while probes **B** and **C** exhibit new near-infrared absorption peaks at 734 nm corresponding to absorption of the hemicyanine moieties with opened spirolactam ring structures at pH 2.5. The hemicyanine absorption peaks of probes **A**, **B**, and **C** show significant increases and undergo slight blue shifts when pH changes from 7.4 to 2.5. A decrease of pH from 7.4 to 2.5 also causes increases of TPE absorption peak of probe **B**, and results in slight decreases of TPE absorption peak of probe **C**. Both hemicyanine absorption peaks of probes **B** and **C** at acidic pH show red shifts by 19 nm compared with that of probe **A** at 715 nm, but there is no significant difference between absorption peaks of hemicyanine moieties of probes **B** and **C**, which consist of TPE and hemicyanine moieties with single and double sp^2 carbon-carbon connections, respectively. These results indicate that probes **B** and **C** show better π -conjugation than probe **A**.

Fluorescence Responses of the Probes to pH Changes

Potential ratiometric fluorescence responses of probes **A**, **B**, and **C** to pH changes were studied in 10 mM citrate buffers with different pH values containing 30% ethanol (Figures 4, 5, and 6). Probe **A** exhibits a fluorescence peak at 510 nm of TPE donor with fluorescence quantum yield of 14.0% at pH 7.4 while probe **B** shows a very strong broad fluorescence peak of TPE moiety at 505 nm with fluorescence quantum yield of 5.58% at pH 7.4, because the hemicyanine moieties of the probes keep their spirolactam ring structures closed at a basic or neutral pH (Figures 3 and 4). Probe **C** displays a very intense fluorescence peak of TPE moiety at 540 nm with fluorescence quantum yield of 7.18% at 7.4 and shows a red shift of 30 nm in TPE emission compared with that of probe **A**, indicating that a vinyl connection between TPE and hemicyanine moieties enhances π -conjugation of the TPE moiety. Decreases of pH from 7.4 to 3.0 induce significant decreases of TPE fluorescence, and increases of the hemicyanine fluorescence under excitation of 420 nm for probe **A**, and 390 nm for probes **B** and **C**, because the hemicyanine moieties undergo spirolactam ring opening with significantly extended π -conjugation at acidic pH (Figures 4–6). Probe **A** shows extraordinary ratiometric responses to pH changes from pH 7.4 to 3.0 with significant ratio increase of hemicyanine fluorescence to TPE fluorescence ($I_{737\text{ nm}}/I_{510\text{ nm}}$) from 0.02 to 4.75 (with a 238-fold remarkable increase) at excitation of 420 nm because the enhanced acidic strength almost causes the disappearance of TPE donor fluorescence, and

considerable increase of hemicyanine acceptor fluorescence (Figure 4 left and right). In addition, probe **A** exhibits well-defined dual fluorescence peaks with a pseudo-Stokes shift of 361 nm (Figure 4, left). Highly sensitive ratiometric responses of probe **A** to pH changes arise from high efficient TBET from the TPE donor to the hemicyanine acceptor because emission of TPE donor shows a very small overlap with absorption of hemicyanine acceptor (Figure S19 in Supporting Information), which prevents efficient FRET from TPE donor to hemicyanine acceptor). Probe **B** also shows good ratiometric response to pH changes from 7.4 to 2.5 with ratio increase of hemicyanine fluorescence over TPE fluorescence ($I_{754\text{ nm}}/I_{505\text{ nm}}$) from 0.03 to 1.7 (with a nearly 57-fold increase), and undergo considerable decrease of TPE moiety fluorescence and substantial increase of hemicyanine moiety fluorescence (Figure 5). Probe **C** displays moderate ratiometric fluorescence responses to pH changes from 7.4 to 2.5 with ratio increase of hemicyanine fluorescence to TPE fluorescence ($I_{747\text{ nm}}/I_{540\text{ nm}}$) from 0.02 to 3.6 (with a roughly 180-fold increase) as it shows complete disappearance of the TPE fluorescence at 540 nm, and relatively small fluorescence increases of the hemicyanine moiety at 747 nm (Figure 6). Probes **A**, **B**, and **C** also show significant near-infrared fluorescence responses to pH changes from 7.4 to 2.5 at excitation of 680 nm, and display molar absorptivity of 2.36×10^4 , 1.51×10^4 , and $1.65 \times 10^4\text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Figures 4–6, right), and fluorescence quantum yields of 6.78%, 2.72%, and 2.84% at pH 4.0, respectively. Both probes **B** and **C** show red shifts in fluorescence peaks of hemicyanine moieties by 17 and 10 nm, respectively, compared with that of hemicyanine acceptor of probe **A** at 737 nm, indicating that probes **B** and **C** possess more extended π -conjugation systems than probe **A**.

Selectivity of the Probes to pH over Metal Ions, Anions, and Amino Acids

The selectivity of the probes to pH over metal ions, anions, and amino acids was investigated by fluorescence spectroscopy. Probes **A**, **B**, and **C** display selective fluorescence responses to pH over 50 μM K^+ , Ca^{2+} , and Mg^{2+} ions which are ubiquitous in mammalian cells, as well as to 50 μM heavy and transitional metal ions such as Ag^+ , Al^{3+} , Co^{2+} , Fe^{2+} , Fe^{3+} , Mn^{2+} , Ni^{2+} , Zn^{2+} , and Cu^{2+} ions (Figures 7, 8, and 9, and S20–S25). In addition, the presence of 50 μM different amino acids and biothiols (such as DL-leucine, DL-cystine, DL-methionine, glycine, DL-proline, DL-alanine, DL-tyrosine, DL-arginine, L-cysteine, and reduced glutathione) shows negligible effects on fluorescence responses of the probes to pH at 7.0 and 2.5 (Figures 7–9 and S20–S25). The presence of 50 μM different anions such as I^- , Br^- , NO_2^- , NO_3^- , SO_3^{2-} , SO_4^{2-} , S^{2-} , HCO_3^- , and CO_3^{2-} also exhibits no influence on fluorescence responses of the probes to pH at 7.0 and 2.5 (Figures 7–9 and S20–S25).

Photostability and Reversibility of the Fluorescent Probes

Photostability of the probes was studied by continuously exciting them at 5 min intervals and evaluating fluorescence intensity every 10 min. Probe **A** shows good photostability with TPE fluorescence decrease by 4.0% and 2.9% in 10 mM citrate buffers with pH 7.0 and 2.5 under excitation of 420 nm, and hemicyanine fluorescence decrease by 3.1% and 6.2% in 10 mM citrate buffer with pH 2.5 under 1 h excitation at 420 and 680 nm, respectively (Figure 10). It displays a TPE fluorescence decrease by 10.5% and 8.9% in 10 mM citrate buffers with pH 7.0 and 2.5 under excitation of 420 nm, and cyanine fluorescence decrease by

11.6% and 13.1% in 10 mM citrate buffer with pH 2.5 under 3 h excitation at 420 and 680 nm, respectively (Figure 10). Fluorescent probes **B** and **C** display similar photostability to probe **A** (Figures S29 and S30). In addition, probes **A–C** display excellent reversible fluorescence responses to pH changes from 7.4 to 2.5 (Figures S26–S28 in Supporting Information).

Cytotoxicity of the Probes

Cytotoxicity was investigated to evaluate the features of the probes for live cell imaging by using a standard MTS assay after 48 h of incubation of HeLa cells with probes **A**, **B**, and **C** at different probe concentrations. Probes **A**, **B**, and **C** display relative low cytotoxicity at high concentrations at 20 μ M with cell visibility of more than 87% (Figure 11).

Fluorescence Imaging of pH in Live Cells

We investigated whether the probes could possess membrane permeability and image the intracellular pH by carrying out cellular imaging with different probe concentrations in HeLa cells. Use of the morpholine residues allows the probes to specifically target acidic lysosomes with pH around 4.5. In order to confirm specific targeting of the probes to acidic lysosomes in live cells, the probes and commercial LysoTracker Red were coincubated with cells in media. Probes **A** and **B** at 20 μ M concentration exhibit both strong fluorescence intensity of TPE and hemicyanine moieties under excitation of TPE moieties at 405 nm, and strong fluorescence intensity of hemicyanine moieties under excitation of hemicyanine moieties at 635 nm (Figures 12 and S31). Probe **C** at 20 μ M concentration shows strong fluorescence intensity of TPE moiety and moderate fluorescence intensity of hemicyanine moiety under excitation of TPE moiety at 405 nm, and strong fluorescence intensity of hemicyanine moiety under excitation of hemicyanine moiety at 635 nm (Figure S32). All probes show excellent membrane permeability and accumulate in the same cellular compartments in live cells with LysoTracker Red because the colocalization analysis of the probes and commercial LysoTracker red based on the Pearson's coefficient gave a value of 0.89 or higher (Figures S33–S35).

After it was demonstrated that the probes could be used to specifically target acidic lysosomes in live cells, they were further evaluated to determine whether they could show ratiometric responses to pH changes in live cells by incubating cells in media with different pH values from 3.0 to 7.0 in the presence of 5 μ g/mL H^+/K^+ ionophore nigericin, which was employed to equilibrate the intracellular and extracellular pH.^{22,27,37,38,42} Probe **A** shows very strong fluorescence of the TPE donor at pH 7.0, and can effectively avoid an issue of blind fluorescence imaging spots at neutral and basic pH values that most intensity-based pH fluorescent probes run into. Moreover, probe **A** displays remarkable ratiometric fluorescence responses to pH changes from pH 7.0 to 3.0 with gradual decreases of the TPE fluorescence and gradual increases of the hemicyanine fluorescence under TPE excitation at 405 nm, since overlapped TPE and hemicyanine fluorescence imaging data under TPE donor excitation undergo significant imaging color changes from deep blue to red in the fourth column in Figure 13. TPE donor fluorescence of probe **A** in live cells completely disappears at pH 3.0 (Figure 13). These observations are in good agreement with fluorescence spectral results in buffer solutions (Figure 4, left). Probe **A** also shows sensitive fluorescence

responses of hemicyanine acceptor to pH changes from 7.0 to 3.0 under hemicyanine excitation at 635 nm, which is in a good agreement with fluorescence observations in buffer solutions (Figure 4, right). In addition, probe **A** also provides dual Stokes shifts with a large pseudo-Stokes shift of 361 nm and dual emissions (Figure 4, left and right), which also allows for colocalization of the imaging readouts of visible and near-infrared fluorescence channels to achieve more precisely double-checked fluorescence ratiometric imaging, since overlapped images of two NIR channels under TPE donor excitation and hemicyanine acceptor excitation change colors from green to yellow in the sixth column of Figure 13, and overlapped images of TPE donor fluorescence under TPE excitation, and hemicyanine acceptor fluorescence under acceptor excitation at 635 nm also undergo significant color changes from deep blue, cyan, and then to deep green in the fifth column of Figure 13, which is in good agreement with fluorescence observations in buffer solutions (Figure 4, right). Probe **B** displays slightly less sensitive ratiometric fluorescence responses to pH changes in live cells than probe **A** (Figure 14). Probe **C** shows much less sensitive ratiometric fluorescence responses to pH changes in live cells than probe **B** (Figure 15). Much more sensitive ratiometric fluorescence responses of probe **A** to pH changes result from highly efficient TBET from TPE donor to hemicyanine acceptor.

Conclusions and Remarks

In summary, we present three ratiometric near-infrared fluorescent probes bearing TPE and hemicyanine moieties with single and double sp^2 carbon–carbon connections based on TBET and π -conjugation modulation strategies. Probes show ratiometric fluorescence responses to pH changes from 7.0 to 3.0 in buffers and in live cells with TPE visible fluorescence decreases and hemicyanine near-infrared fluorescence increases. However, probe **A** based on the TBET strategy displays more sensitive ratiometric fluorescence responses to pH changes than probes **B** and **C** based on the π -conjugation modulation strategy. Probe **A** shows large signal-to-background fluorescence ratio changes up to a 238-fold in ratio of the visible TPE fluorescence intensity to hemicyanine fluorescence intensity in response to pH changes from 7.4 to 3.0. The platform of probe **A** will allow for construction of a variety of ratiometric fluorescent probes for ratiometric detection of metal ions, biological thiols, reactive nitrogen, and oxygen species by incorporating different sensing ligands to the hemicyanine acceptor to form on–off spirolactam structures.

Materials and Methods

Materials

Unless specifically indicated, all reagents and solvents were purchased from commercial suppliers and used without further purification. ^1H NMR and ^{13}C NMR spectra in CDCl_3 and CD_3OD solutions were collected by 400 MHz Varian Unity Inova NMR spectrophotometer instrument. Solvent residual peaks (^1H : δ 7.26 for CDCl_3 , δ 3.31 for CD_3OD ; ^{13}C : δ 77.3 for CDCl_3 , δ 39.9 for CD_3OD) were used as internal standards in ppm to determine chemical shifts (δ) of intermediates and probes. High-resolution mass spectrometer data (HRMS) were measured by using double focusing magnetic mass spectrometer, fast atom bombardment (FAB) ionization mass spectrometer, or matrix

assisted laser desorption/ionization time-of-flight mass spectrometer. Absorption and fluorescence spectra were conducted by using Perkin Elmer Lambda 35 UV/vis spectrometer and Jobin Yvon Fluoromax-4 spectrofluorometer, respectively.

Probe A

The solution of compound **7a** (100 mg, 0.14 mmol) and compound **8** (94 mg, 0.18 mmol) in a mixture solvent of toluene/ethanol (10/4 mL) was bubbled for 10 min under N₂. When tetrakis(triphenylphosphine)palladium (13 mg, 18 μ mol) and Na₂CO₃ (aq, 0.2 mL) were added, the mixture was heated at 90 °C for 24 h under N₂. The solvent was evaporated under vacuum. The residue was added with water and extracted with dichloromethane (2 $\times$ 20 mL). The organic layers were collected, dried over Na₂SO₄, filtered, and evaporated. The crude was purified by flash column chromatography using dichloromethane and methanol (20:1, v/v) to give probe **A** (55 mg, 38%). ¹H NMR (400 MHz, CDCl₃) δ : 8.03 (s, 1H), 7.65 (d, *J* = 8.0 Hz, 2H), 7.46 (d, *J* = 11.2 Hz, 2H), 7.39 (d, *J* = 8.0 Hz, 2H), 7.16–7.04 (m, 9H), 6.95 (t, *J* = 9.2 Hz, 4H), 6.83 (t, *J* = 7.2 Hz, 1H), 6.65–6.59 (m, 4H), 6.42 (d, *J* = 8.8 Hz, 1H), 6.35 (d, *J* = 2.0 Hz, 1H), 6.31 (d, *J* = 8.8 Hz, 1H), 5.36 (d, *J* = 12.8 Hz, 1H), 3.73 (s, 3H), 3.72 (s, 3H), 3.60 (t, *J* = 4.0 Hz, 4H), 3.45–3.39 (m, 1H), 3.33–3.26 (m, 1H), 3.14 (s, 3H), 2.96 (s, 6H), 2.62–2.57 (m, 1H), 2.46–2.34 (m, 7H), 2.24–2.17 (m, 2H), 1.72 (s, 3H), 1.71 (s, 3H), 1.64–1.59 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ : 168.3, 158.3, 157.9, 152.6, 151.4, 150.4, 148.1, 145.5, 144.3, 144.0, 141.2, 140.7, 139.0, 138.8, 137.7, 136.4, 132.7, 132.1, 131.6, 131.0, 128.7, 127.9, 126.5, 126.3, 123.7, 121.7, 121.1, 120.4, 119.7, 119.5, 113.3, 113.2, 109.0, 106.6, 105.9, 103.9, 98.6, 92.2, 67.2, 66.8, 56.7, 55.4, 53.8, 45.7, 40.7, 37.2, 29.4, 28.8, 28.7, 25.7, 23.4, 22.6. HRMS (ESI): calculated for C₆₉H₆₉N₄O₅⁺ [M + H]⁺ 1033.5190, found 1033.5273.

Probe B

The solution of compound **14a** (98 mg, 0.11 mmol) and compound **8** (80 mg, 0.15 mmol) in THF (10 mL) was bubbled for 10 min under N₂. After tetrakis(triphenylphosphine)palladium (13 mg, 11 μ mol) and Na₂CO₃ (aq, 0.2 mL) were added, the mixture was heated at 90 °C for 24 h under N₂. The solvent was evaporated under vacuum. The residue was added with water and extracted with dichloromethane (2 $\times$ 20 mL). The organic layers were collected, dried over Na₂SO₄, filtered, and evaporated. The crude was purified by flash column chromatography using dichloromethane and methanol (10:1, v/v) to give probe **B** (33 mg, 26%). ¹H NMR (400 MHz, CDCl₃) δ : 7.83 (d, *J* = 6.8 Hz, 1H), 7.53–7.50 (m, 1H), 7.44–7.40 (m, 2H), 7.32–7.27 (m, 4H), 7.15 (d, *J* = 7.6 Hz, 1H), 7.11–6.97 (m, 9H), 6.95 (d, *J* = 8.8 Hz, 2H), 6.72 (d, *J* = 8.4 Hz, 1H), 6.66–6.61 (m, 4H), 6.33–6.29 (m, 2H), 6.24–6.22 (m, 1H), 5.48 (d, *J* = 12.8 Hz, 1H), 3.83–3.79 (m, 2H), 3.73 (s, 3H), 3.72 (s, 3H), 3.71–3.69 (m, 2H), 3.60–3.59 (m, 2H), 3.41–3.38 (m, 1H), 3.35–3.30 (m, 4H), 3.27–3.23 (m, 1H), 2.94 (t, *J* = 7.6 Hz, 2H), 2.59–2.53 (m, 5H), 2.42–2.35 (m, 5H), 2.25–2.22 (m, 2H), 1.71 (s, 3H), 1.70 (s, 3H), 1.64–1.59 (m, 2H), 1.15 (t, *J* = 7.2 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃) δ : 168.5, 158.2, 156.6, 153.0, 151.8, 148.8, 148.2, 144.6, 142.4, 140.1, 139.5, 139.1, 136.7, 132.8, 132.2, 132.0, 131.7, 128.9, 128.3, 127.9, 126.6, 126.3, 125.6, 123.6, 123.0, 120.4, 120.2, 119.7, 113.3, 113.2, 108.5, 106.7, 105.4, 103.8, 97.9, 92.9, 67.2, 66.8, 56.6, 54.1, 53.7, 49.4, 45.8, 44.6, 37.0, 28.7, 25.5, 22.8, 22.4, 12.8. HRMS (ESI): calculated for C₇₃H₇₅N₄O₈S[−] [M][−] 1167.5306, found 1167.5314.

Probe C

Compound **15** (63 mg, 0.15 mmol), palladium acetate (2 mg), tri(*o*-tolyl)phosphine (6 mg, 20 μ mol), and Et₃N (3 mL) were added to the solution of compound **14b** (80 mg, 0.1 mmol) in DMF (6 mL). The mixture was heated at 90 °C under argon overnight. After completion of the reaction (monitored by LC-MS), the reaction was concentrated under reduced pressure. The residue was purified by flash column chromatography using dichloromethane and methanol (20:1, v/v) as the eluent to give probe **C** (35 mg, 32%). ¹H NMR (400 MHz, CDCl₃) δ : 7.84 (d, *J* = 6.8 Hz, 1H), 7.50–7.39 (m, 6H), 7.32–7.28 (m, 3H), 7.22 (d, *J* = 8.0 Hz, 1H), 7.16 (d, *J* = 7.2 Hz, 1H), 7.11–7.05 (m, 6H), 6.98–6.96 (m, 3H), 6.93 (d, *J* = 8.8 Hz, 1H), 6.67–6.61 (m, 3H), 6.55 (d, *J* = 8.0 Hz, 1H), 6.34–6.31 (m, 2H), 6.26–6.23 (m, 1H), 5.38 (d, *J* = 12.8 Hz, 1H), 5.30 (d, *J* = 18.0 Hz, 1H), 3.74 (s, 3H), 3.72 (s, 3H), 3.60 (t, *J* = 4.4 Hz, 4H), 3.42–3.40 (m, 1H), 3.35–3.28 (m, 5H), 3.15 (s, 3H), 2.62–2.57 (m, 2H), 2.44–2.34 (m, 6H), 2.24–2.20 (m, 2H), 1.74 (s, 3H), 1.73 (s, 3H), 1.63–1.58 (m, 2H), 1.16 (t, *J* = 6.8 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃) δ : 168.5, 158.3, 157.6, 153.0, 151.7, 148.9, 148.1, 145.3, 144.5, 143.3, 140.3, 139.6, 139.2, 136.6, 135.8, 132.8, 132.2, 131.9, 131.7, 129.2, 128.3, 127.9, 126.3, 125.6, 124.4, 123.6, 123.0, 121.0, 120.0, 119.3, 113.3, 113.2, 108.6, 105.9, 105.5, 104.1, 97.9, 92.9, 67.2, 67.0, 56.7, 55.3, 53.7, 45.5, 44.6, 37.1, 29.5, 28.5, 25.6, 24.8, 23.2, 22.4, 12.7. HRMS (ESI): calculated for C₇₃H₇₅N₄O₅⁺ [M + H]⁺ 1087.5659, found 1087.5636.

Cell Culture and Cytotoxicity Assay

HeLa cells were purchased from ATCC (Mannassas, VA) and were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Fisher Scientific) at 37 °C in humidified air and 5% CO₂. The cells were subcultured at 90% confluence with 0.25% trypsin (w/v) every 2–3 days. Standard MTS assay was used to investigate the cytotoxicity of probes against HeLa. The cells were seeded in 96-well plates at an initial density of 3000 cells per well, with 100 μ L DMEM medium per well. After seeding 24 h on the 96 well plate, the medium was replaced by different concentrations of probes **A**, **B**, and **C** (0, 5, 10, 15, and 20 μ M concentration solutions in fresh culture medium, 100 μ L/well) for 48 h at 37 °C under 5% CO₂. The probe solutions were replaced with the fresh culture medium (80 μ L/well), and CellTiter 96 Aqueous (20 μ L/well) was added to evaluate cell viability. After incubation for another 2 h, the cell viability was determined by measuring the light absorbance at 490 nm with a microplate reader (BioTek ELx800). Untreated cells were employed as controls. Percent (%) cell viability was calculated by comparing the absorbance of the control cells to that of treated cells. Data were summarized as a plot where each data point represents an average of parallel three wells.

Fluorescence Cell Image of the Probes in Different pH

For confocal live cell imaging, HeLa cells were seeded into the 35 nm glass-bottom culture dishes (MatTek, MA) and allowed 1 day to reach 50% confluence. After 24 h of incubation, the cell culture medium was replaced by freshly prepared FBS-free medium with 5, 10, 15, and 20 μ M of probe **A** or 5, 10, and 20 μ M of probes **B** and **C** for 1 h 37 °C under 5% CO₂ followed by using 50 nM LysoTracker red (Thermo-Fisher) for 30 min to confirm the specific

targeting of our probes to lysosomes in HeLa cells. The cells were rinsed with PBS buffer twice before imaging. For the live cell fluorescence imaging at different pH values, the HeLa cells were treated with 15 μ M probes **A**, **B**, and **C** at 37 °C under 5% CO₂ for 1 h. The cells were rinsed with PBS buffer twice before they were treated with nigericin (5 μ g/mL) in citric buffer with pH values at 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 for probes **A** and **B** or at 2.5, 3.5, 4.5, 5.5, 6.5, and 7.5 for probe **C** to equilibrate the intracellular and extracellular pH for 30 min. The cells were rinsed with PBS buffer twice again before imaging. Live cell images were taken by a confocal fluorescence microscope (Olympus IX 81). The excitation wavelength of the TPE donor was at 405 nm and the fluorescence images were collected from 425 to 475 nm for blue channel, and from 725 to 775 nm for red NIR channel. The excitation wavelength of the hemicyanine acceptor is at 635 nm, and the fluorescence images were collected from 725 to 775 nm for green NIR channel. The excitation wavelength of LysoTracker red is at 559 nm, and the fluorescence images were collected from 580 to 620 nm (orange channel).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fluorescence spectra of 5 μM probes **A** (left), **B** (middle), and **C** (right) in response to water percentage changes from 0% to 99% in mixed water and ethanol solutions under excitation of 420, 390, and 390 nm for probes **A**, **B**, and **C**, respectively.

Figure 2.

Fluorescence photo of 5 μM probe **A** in water and ethanol mixed solutions with different water percentages from 0% to 99% under radiation of a UV lamp.

Figure 3.

Absorbance spectra of 5 μM probes **A** (left), **B** (middle), and **C** (right) in 10 mM citrate buffers with different pH values containing 30% ethanol.

Figure 4.

Fluorescence spectra of 5 μM probe **A** in 10 mM citrate buffers with different pH values containing 30% ethanol under excitation of 420 nm (left), 420 nm (right), and 680 nm (right), respectively; pH dependent fluorescence ratios of hemicyanine acceptor ($I_{737\text{ nm}}$) to TPE donor ($I_{510\text{ nm}}$) under excitation of 420 nm (middle).

Figure 5.

Fluorescence spectra of 5 μM probe **B** in 10 mM citrate buffers with different pH values containing 30% ethanol under excitation of 390 nm (left), 390 nm (right), and 680 nm (right), respectively; pH dependent fluorescence ratios of hemicyanine moiety ($I_{754 \text{ nm}}$) to TPE moiety ($I_{505 \text{ nm}}$) under excitation of 390 nm (middle).

Figure 6.

Fluorescence spectra of 5 μM probe **C** in 10 mM citrate buffer containing 30% ethanol under excitation of 390 nm (left), 390 nm (right), and 680 nm (right), respectively; pH dependent fluorescence ratios of hemicyanine moiety ($I_{747\text{ nm}}$) to TPE moiety ($I_{540\text{ nm}}$) under excitation of 390 nm (middle).

Figure 7.

TPE fluorescence responses of 5 μM probe A at 510 nm to 50 μM metal ions (left), 50 μM anions (middle), and amino acids (right) in 10 mM citrate buffers with pH 7.0 and 2.5 under excitation of 420 nm.

Figure 8.

Hemicyanine fluorescence responses of 5 μM probe A at 737 nm to 50 μM metal ions (left), 50 μM anions (middle), and amino acids (right) in 10 mM citrate buffers with pH 7.0 and 2.5 under excitation of 420 nm.

Figure 9. Hemicyanine fluorescence responses of 5 μ M probe A at 737 nm to 50 μ M metal ions (left), 50 μ M anions (middle), and amino acids (right) in 10 mM citrate buffers with pH 7.0 and 2.5 under excitation of 680 nm.

Figure 10.

TPE fluorescence intensity of 5 μ M probe A at 510 nm in 10 mM citrate buffers with pH 7.0 and 2.5 under excitation at 420 nm (right), and hemicyanine fluorescence intensity of 5 μ M probe A at 737 nm in 10 mM citrate buffer with pH 2.5 under excitation at 420 and 680 nm (left).

Figure 11.

Cytotoxicity of probes **A**, **B**, and **C** tested by MTS assay. The HeLa cells were incubated with 5, 10, 15, and 20 μM of probes **A**, **B**, and **C** for 48 h. The absorbance measured at 490 nm is directly proportional to the cell viability. The error bars indicate $\pm$ SD.

Figure 12.

Fluorescence images of HeLa cells incubated with Lysotracker Red and different concentrations of probe **A**. Images were obtained by the confocal fluorescence microscope at 60 $\times$ magnification. The excitation of TPE donor was under 405 nm, and the images of blue channel in the first column was collected from 425 to 475 nm, while red NIR channel in the second column was collected from 725 to 775 nm. The excitation of hemicyanine acceptor was under 635 nm, and green NIR channel in the third column was collected from 725 to 775 nm. The excitation of lysotracker red was at 559 nm and orange channel in the fourth column was collected from 580 to 620 nm. Scale Bar: 50 μ M.

Figure 13.

Fluorescence images of HeLa cells incubated with 15 μM probe **A**. HeLa cells were incubated with 15 μM probe **A** in 10 mM citrate buffers with pH from pH 3.0 to 7.0 in the presence of 5 $\mu\text{g/mL}$ nigericin. Images were acquired using the confocal fluorescence microscope at 60 $\times$ magnification. The excitation of TPE donor was at 405 nm, and the blue channel in the first column was collected from 425 to 475 while the red NIR channel in the second column was collected from 725 to 775 nm. The excitation of hemicyanine acceptor was at 635 nm, and the green NIR channel in the third column was collected from 725 to 775 nm. Scale bar: 50 μM .

Figure 14.

Fluorescence images of HeLa cells incubated with 15 μM probe **B**. HeLa cells were incubated with 15 μM probe **B** in 10 mM citrate buffers with pH from pH 3.0 to 7.0 in the presence of 5 $\mu\text{g/mL}$ nigericin. Images were acquired using the confocal fluorescence microscope at 60 $\times$ magnification. The excitation of TPE moiety was at 405 nm, and the blue channel in the first column was collected from 425 to 475 nm, while the red NIR channel in the second column was collected from 725 to 775 nm, respectively. The excitation of hemicyanine moiety was at 635 nm, and the green NIR channel in the third column was collected from 725 to 775 nm. Scale bar: 50 μM .

Figure 15.

Fluorescence images of HeLa cells incubated with 15 μM probe **C**. HeLa cells were incubated with 15 μM probe **C** in 10 mM citrate buffers with pH from pH 2.5 to 7.5 in the presence of 5 $\mu\text{g/mL}$ nigericin. Images were acquired using the confocal fluorescence microscope at 60 $\times$ magnification. The excitation of TPE moiety was at 405 nm, and the blue channel in the first column was collected from 425 to 475 nm, and the red NIR channel in the second column was collected from 725 to 775 nm, respectively. Scale bar: 50 μM .

Scheme 1. Chemical Structure Responses of Ratiometric Near-Infrared Fluorescent Probes to pH Changes

Scheme 2. Synthetic Route to Prepare Ratiometric Fluorescent Probes A, B, and C Consisting of TPE and Hemicyanine Moieties