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New Near-infrared Fluorescent Probes with Single-photon Anti-Stokes-shift Fluorescence for Sensitive Determination of pH Variances in Lysosomes with a Double-Checked Capability

Tzu-Ho Chen^{a,d}, Shuwei Zhang^{b,e}, Meghnath Jaishi^c, Rashmi Adhikari^b, Jianheng Bi^b, Mingxi Fang^b, Shuai Xia^b, Yibin Zhang^a, Rudy L. Luck^a, Ranjit Pati^c, Hsien-Ming Lee^a, Fen-Tair Luo^a, Ashutosh Tiwari^b, and Haiying Liu^b

^aInstitute of Chemistry, Academia Sinica, Taipei, Taiwan 11529, Republic of China.

leehm1@chem.sinica.edu.tw and luoft@gate.sinica.edu.tw

^bDepartment of Chemistry, Michigan Technological University, 1400 Townsend Drive, Houghton, MI 49931, USA. tiwari@mtu.edu; hylu@mtu.edu

^cDepartment of Physics, Michigan Technological University, 1400 Townsend Drive, Houghton, MI 49931, USA. patir@mtu.edu

^dDepartment of Chemistry, National Taiwan University, No. 1 Roosevelt Road Section 4, Taipei 10617, Taiwan, Republic of China

^eSchool of Chemistry and Chemical Engineering, Yangzhou University, Yangzhou 225002, China.

Abstract

Two near-infrared luminescent probes with Stokes-shift and single-photon anti-Stokes-shift fluorescence properties for sensitive determination of pH variance in lysosomes have been synthesized. A morpholine residue in probe **A** which serves as a targeting group for lysosomes in viable cells was attached to the fluorophores via a spirolactam moiety while a mannose residue was ligated to probe **B** resulting in increased biocompatibility and solubility in water. Probes **A** and **B** contain closed spirolactam moieties, and show no Stokes-shift or anti-Stokes-shift fluorescence under neutral or alkali conditions. However, the probes incrementally react to pH

Corresponding authors: leehm1@chem.sinica.edu.tw; luoft@gate.sinica.edu.tw; patir@mtu.edu; tiwari@mtu.edu; hylu@mtu.edu.

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ASSOCIATED CONTENT

The supporting information can be obtained on the ACS publication website. Supporting information includes instrumentation, syntheses of intermediates, NMR spectra, and intracellular images, determination of fluorescence quantum yields, pK_a values of the probes, theoretical computation of the probes, photostability of the probes, the probe selectivity and reversibility to pH changes, MTS assay, and fluorescence cellular imaging obtained by using AMF-4306, EVOSfl, AMG inverted fluorescence microscopy, Zeiss LSM 510 confocal microscope and Olympus IX71 inverted fluorescence microscopy.

variance from 7.22 to 2.76 with measurable increases in both Stokes-shift and anti-Stokes-shift fluorescence at 699 nm and 693 nm under 645 nm and 800 nm excitation, respectively. This acid-activated fluorescence is produced by the breaking of the probe spirolactam moiety, which greatly increased overall π -conjugation in the probes. These probes possess upconversion near-infrared fluorescence imaging advantages including minimum cellular photo-damage, tissue penetration, and minimum biological fluorescence background. They display excellent photostability with low dye photobleaching and show good biocompatibility. They are selective and capable of detecting pH variances in lysosomes at excitation with two different wavelengths, i.e., 645 and 800 nm.

TOC Graphic

Keywords

Near-infrared imaging; fluorescent probes; single-photon anti-Stokes-shift fluorescence; acidic pH in lysosome; theoretical computation

1. INTRODUCTION

A stable pH inside living cells is important to maintain cellular homeostasis such as cellular proliferation, metabolism, phagocytosis, and enzymatic activity.^{1–3} Mitochondria function under slightly basic conditions (pH 8.0) while lysosomes work best under acidic pH conditions (pH 4.5 to 5.0) so that approximately 70 hydrolytic enzymes can break down a variety of biological macromolecules.^{4–6} Abnormal intracellular pH values are linked to cellular dysfunction and numerous diseases including cancer, cardiopulmonary, neurodegenerative diseases including Parkinson disease and Alzheimer's disease.^{7–8} The extracellular fluid pH value of cancer cells and tissues range from 6.2 to 6.9, which is significantly lower than the physiological pH (7.4) of normal cells.⁹ Therefore, accurate detection of intracellular pH is vital in order to assess the health of living cells and cellular internalization pathways. Previous studies have been directed at the detection of intracellular pH using NMR, electrochemical microelectrodes,¹⁰ and fluorescence microscopy methods.^{11–16} Among these methods, fluorescent probes in conjunction with fluorescence

microscopy for pH imaging have desirable advantages including operational ease, non-invasive and simultaneous results, high sensitivity, and high space-time resolution.^{2–3, 11–23} Most of the previously reported fluorescent probes constructed to monitor intracellular pH, absorb and emit at less than 600 nm wavelength. This can potentially cause cellular and tissue photodamage, and have biological autofluorescence issues.^{2–3, 24} Many near-infrared (NIR) fluorescent probes were reported to address these issues.^{11–16, 18, 23, 25–32} However, most of NIR fluorescent probes are based on Stokes-shift fluorescence with lower energy emission under higher energy excitation.^{2–3, 11–19} Recently, a small organic molecule with single-photon anti-Stokes-shift fluorescence was reported to be useful for Hg(II) and Cu(II) detection.^{33–34} We reported on probes featuring anti-Stokes-shift fluorescence, with NIR emission at 740 nm under excitation at 800 nm for detection of intracellular pH changes.¹⁸ These fluorescent probes with one-photon anti-Stokes-shift properties have NIR imaging advantages which effectively negate dye photo bleaching issues under NIR excitation.¹⁸ Nevertheless, organic fluorophores with single-photon anti-Stokes-shift properties are still extremely underdeveloped and limited to molecules with one-photon NIR excitation and emission capability.¹⁸ For reasons alluded to above, it is very important to expand NIR fluorophores with one-photon anti-Stokes-shift properties to take advantage of upconversion near-infrared fluorescence imaging (Scheme 1). In this article, we report on the synthesis of two fluorescent probes with single-photon anti-Stokes-shift fluorescence properties. Probe **A** contains a lysosome-targeting morpholine residue for sensitive determination of pH variance in lysosomes with double-checked capability through Stokes-shift and anti-Stokes-shift NIR fluorescence channels (Schemes 1 and 2). We introduced a mannose residue to probe **B** to improve probe biocompatibility and solubility in aqueous solutions. The probes do not display Stokes-shift and anti-Stokes-shift fluorescence under neutral or alkali pH conditions. However, they respond sharply to pH changes from 7.22 to 2.76 with increases in both Stokes-shift and one-photon anti-Stokes-shift fluorescence for probes **A** at 699 nm, and **B** at 693 nm under excitation at either 645 nm or 800 nm. This is because acidic pH conditions result in the opening of the fluorophore spirolactam moiety in the neutral probes. Absorption peaks obtained by theoretical modeling of the ring opened probes match well with our experimental data. These molecules were successfully applied to detect lysosomal pH variance.

2. EXPERIMENTAL SECTION

Synthesis of probe A.

Compound **5** (100.0 mg, 0.17 mmol), N,N'-dicyclohexylcarbodiimide (DCC) (41.1 mg, 0.20 mmol) and N-hydroxysuccinimide (24.8 mg, 0.22 mmol) were mixed in methylene chloride (5 mL). The solution was agitated at ambient temperature for 30 minutes followed by adding 3-morpholinopropylamine (**6**) (46.9 mg, 0.33 mmol) and the mixture allowed to react for 2 h. After removal of the solvent, the product was obtained as a brown solid by preparative TLC employing as the eluant a solution of dichloromethane and methanol in a ratio of 20 to 1 respectively (79.0 g, 75.8%). ¹H NMR (400 MHz, CDCl₃) δ 8.72 (s, 1H), 7.90–7.85 (m, 2H), 7.54 (s, 1H), 7.49–7.39 (m, 3H), 7.31–7.18 (m, 4H), 6.42 (d, *J* = 4.0 Hz, 1H), 6.34 (d, *J* = 8.0 Hz, 1H), 6.28–6.25 (m, 1H), 3.61 (t, *J* = 4.0 Hz, 3H), 3.37–3.31 (m, 4H), 3.28–3.21 (m, 1H), 2.83–2.63 (m, 2H), 2.29–2.17 (m, 5H), 1.71–1.46 (m, 6H), 1.17 (t, *J* = 8.0 Hz, 6H);

^{13}C NMR (100 MHz, CDCl_3) δ 168.6, 153.1, 151.5, 148.9, 147.7, 135.9, 132.4, 132.3, 128.9, 128.4, 128.0, 127.2, 123.8, 123.6, 123.0, 122.9, 120.3, 119.3, 115.1, 113.9, 111.4, 108.6, 106.8, 105.3, 97.9, 67.2, 66.9, 56.5, 53.6, 44.5, 38.4, 28.6, 25.3, 23.1, 22.5, 12.9. HRMS (ESI): calculated for $\text{C}_{40}\text{H}_{45}\text{N}_4\text{O}_3^+$ $[\text{M}+\text{H}]^+$, 629.3492; found, 629.3483.

Synthesis of probe B.

K_2CO_3 (60.2 mg, 0.44 mmol) and compound **10** (0.11 g, 0.11 mmol) were added to methanol (6 mL), and stirred at ambient temperature for 2 h. The salt was separated from the solution and the filtrate concentrated, affording probe **B** as a light brown solid (82.0 mg, 89.6%). ^1H NMR (400 MHz, CD_3OD) δ 7.82–7.80 (m, 2H), 7.64 (d, J = 8.0 Hz, 1H), 7.55–7.46 (m, 2H), 7.41–7.38 (m, 2H), 7.21–7.17 (m, 1H), 7.14–7.07 (m, 2H), 6.57–6.55 (m, 1H), 6.27 (d, J = 8.0 Hz, 1H), 6.19 (d, J = 8.0 Hz, 1H), 4.68 (d, J = 8.0 Hz, 1H), 4.39–2.29 (m, 2H), 4.04–3.99 (m, 1H), 3.76–3.57 (m, 5H), 3.50–3.42 (m, 4H), 3.31–3.28 (m, 2H), 3.12–3.03 (m, 2H), 2.87–2.83 (m, 2H), 2.65–2.59 (m, 2H), 2.08–1.97 (m, 6H), 1.66–1.23 (m, 8H), 1.09 (t, J = 8.0 Hz, 6H); ^{13}C NMR (150 MHz, CD_3OD) δ 170.4, 161.5, 164.5, 158.1, 152.9, 150.6, 149.6, 137.7, 134.0, 133.3, 130.1, 129.9, 127.2, 124.9, 123.5, 121.1, 120.0, 116.5, 113.7, 111.0, 106.8, 106.5, 102.0, 99.0, 74.8, 72.8, 72.2, 69.0, 68.3, 67.9, 67.8, 62.5, 57.3, 54.5, 50.0, 47.3, 45.5, 39.5, 29.7, 26.1, 24.2, 23.5, 13.1. HRMS (ESI): calculated for $\text{C}_{48}\text{H}_{57}\text{N}_4\text{O}_9^-$ $[\text{M}-\text{H}]^-$, 833.4126; found, 833.4120.

Stokes-shift fluorescence and anti-Stokes-shift fluorescence imaging of intracellular pH changes.

HeLa, KB (a subline of the ubiquitous KERATIN-forming tumor cell line HeLa), MDA-MB231 human breast cancer cell line and HUVEC-C cells (human umbilical vein endothelial cells) were obtained from ATCC. The HeLa cells with a minimum of five passages were put in 12-well culture plates with a density of 10 000 cells/ cm^2 and preserved in an eagle medium based on Dulbecco's modification with 20% fetal bovine serum. Freshly prepared serum-free medium containing probe **A** or **B** (20 or 50 μM concentrations) was used to replace the cell culture medium after 16 h incubation, we further stained the cells with 1 g/L Hoechst for 5 min and LysoTracker Green (50 nM) for 30 min in order to mark the nucleus location and demonstrate whether probes can specifically target lysosomes. Probe **A** or **B** (10 μM) was initially added to the cells at 37 $^\circ\text{C}$ for 30 min followed with incubation of 1 mg/L Hoechst and 50 nM LysoTracker green for 30 min and 5 min, respectively, in order to test whether the probes could identify pH variance in lysosomes. Once the cells were washed by a neutral PBS buffer twice, the cells were put in sodium phosphate buffers with pH ranging from 2.5, 3.5, 4.5, 5.5, 6.5 to 7.5 accompanied with 5 mg/L nigericin for 30 min to homologize the extracellular pH and buffer pH. A Zeiss LSM510 confocal fluorescence microscope and a lamp-based Olympus IX-71 inverted fluorescence microscope were employed to collect cellular fluorescence imaging with both Stokes-shift and single-photon anti-Stokes-shift fluorescence. Suitable excitation wavelengths and each filter constant exposure time were employed to collect LysoTracker Green fluorescence, Stokes-shift fluorescence and anti-Stokes-shift fluorescence images in the same view field to achieve simultaneous localization visualization of our probes and LysoTracker Green. The JACoP plugin from ImageJ was used to obtain Pearson's coefficient in order to accomplish the fluorescence colocalization analysis and determine whether our

probes stay in the same lysosomes with LysoTracker Green. For details on cell culture and Stoke-shift fluorescence cellular imaging of MDAMB231 and HUVEC-C cells, please see supplemental section (Figures S27-S38).

3. RESULTS AND DISCUSSION

The probe synthesis

The reaction of 2-(4-(diethylamino)-2-hydroxybenzoyl)benzoic acid (**1**) with cyclohexanone (**2**) in sulfuric acid afforded compound **3**.^{15, 18, 35–36} Compound **3** was then reacted with indole-3-carboxaldehyde (**4**) in acetic anhydride solution (Scheme 3) yielding the near-infrared fluorescent dye (**5**). Probe **A** was prepared by reacting fluorescent dye **5** first with N-hydroxysuccinimide under dicyclohexylcarbodiimide (DCC) for 30 minutes and then with 3-morpholinopropylamine (**6**) (Scheme 3). To improve biocompatibility and increase aqueous solubility of probe **B**, we ligated a mannose residue to fluorescent dye (**9**) by reacting indole-3-carboxaldehyde bearing a protected mannose residue (**8**) with compound **3**, resulting in residue (**9**). Probe **B** bearing this mannose residue was then prepared by binding a morpholine residue to dye **9**, forming **10**, and then mild de-acetylation of the protected mannose residue from intermediate **10** in methanol with K_2CO_3 .

Probe absorption spectra upon pH changes.

Peaks at 336 nm and 328 relating to absorption by the probe's indole groups were observed for probes **A** and **B** under neutral or alkali pH solutions, respectively (Figure 1 and Scheme 1). However, upon gradual pH decrease from 7.22 to 2.76, new peaks at 649 nm for **A** and 641 nm for **B** were observed. Under acidic conditions, the spirolactam moieties open resulting in extended π -conjugation in the molecules (Figure 1, and Scheme 1).

Stokes-shift and anti-stokes-shift spectra of the probes upon pH changes

The probes reveal very weak fluorescence under neutral or basic pH conditions. However, gradual pH decreases from 7.2 to 2.8 results in Stokes-shift and anti-Stokes-shift fluorescence increases for probes **A** and **B** at 699 nm and 693 nm under excitation at 645 nm and 640 nm, respectively (Figures 2 and S13) Here the fluorophore spirolactam rings open under acidic pH conditions, resulting in significantly enhanced π -conjugation of the probe. Additionally, anti-Stokes-shift fluorescence responses of the probes under one-photon excitation at 800 nm are observed (Figure 3). The probes show Stokes-shift fluorescence quantum yields of 12% and 9.0%, and anti-Stokes-shift fluorescence quantum yields of 2.5% and 2.1%, and have molar absorptivities of 1.3×10^4 and $7.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 2.8, respectively. The Henderson–Hasselbach³⁷ equation was utilized to determine probe pK_a values involving spirolactam ring opening and resulted in pK_a values of 4.80 and 4.40 for probe **A** and **B**, respectively (Scheme 1, Figures 3 and S14). The probes reversibly respond to pH variance ranging from 2.4 to 7.2 for both Stokes-shift and anti-Stokes-shift fluorescence (Figures S21 and S22).

DFT Modeling.

To understand the probe near-infrared fluorescence phenomena, we recourse to a first-principles density functional (DFT) approach.^{38–39} The geometry optimizations of probes **A** and **B** with configurations having closed and opened spirolactam ring were conducted by employing Becke's three parameter hybrid density functional method (B3LYP) incorporating part of the exact Hartree-Fock exchange.⁴⁰ Our calculation is conducted using the 6–311G** gaussian basis set and the polarizable continuum model to represent the solvent effect.⁴⁰ The closed spirolactam moiety configuration of the probes was calculated with water as the solvent. We used formic acid to mimic acidic pH for the spirolactam moiety opened structures. As the protonation of probes **A** and **B** in an acidic environment opens the probe spirolactam rings via bond breaking between the nitrogen atom in the spirolactam ring and the carbon atom in the middle ring of the rhodamine core, a perchlorate (ClO_4^-) counter ion is added to balance the charge for the open ring configuration. Our calculations reveal that the opening of the spirolactam ring is favored by the transfer of charge from the C-N bond of the spirolactam ring to the central ring of the rhodamine core. The charge on the carbon atom before and after the spirolactam ring opening is found to be ~ -0.04 e and ~ 0.19 e, respectively. The delocalization of the charge is found to shorten the C-C bond distance in the rhodamine middle ring from ~ 1.50 Å to ~ 1.40 Å, thereby making the core more conducive to electron delocalization. Figure 4 shows the optimized structures of different two representative probes.

To further elucidate the effect of the spirolactam ring opening on the electronic structure of the probes, we analyzed the frontier energy levels of the probes before and after ring opening. The frontier energy levels along with the HOMO and LUMO of both probes under basic and acidic conditions are shown in Figures S15-S17. Our analysis reveals the HOMO-LUMO energy gaps decrease upon protonation of probes **A** and **B**, which indicate shifts toward the near infrared region of the spectra. To better understand the observed shift in the absorption spectrum, we executed excitonic calculations with time-dependent density functional theory (DFT)^{41–42}; the ground state optimized geometry of the probes were considered for this study. These calculations provide us with various excited states along with the corresponding oscillatory strength that is related to the experimentally measured absorbance. As no prior information is available on the choice of exchange-correlation functional to accurately determine optical properties in different environments (with different pH levels), we performed the excitonic calculations of the probes using three different exchange-correlation functionals. First we did the TD-DFT^{41–42} calculation using the SVWN⁴⁰ functional that utilizes the local density approach (LDA)⁴⁰; S represents the Slater exchange and VWN adds the local correlation. We also utilized the PW91⁴⁰ functional that employs a generalized gradient corrected approach (GGA)⁴⁰ to perform the excitonic calculation. Finally, we performed the time-dependent DFT^{41–42} calculation using the orbital-dependent hybrid B3LYP functional.⁴⁰ A comparative analysis of the results obtained from these different functionals to the experimental observations demonstrates which functionals yield better results under neutral environments and which one works well under acidic environments for these molecules. A Lorentzian function with a half-height width value of 10 nm was used to fit the absorption spectra of all the probes (Figure 5). Our results show that at neutral pH when both probes have their spirolactam ring closed, the

hybrid functional B3LYP⁴⁰ that includes a fraction of the exact Hartree-Fock⁴³ exchange calculates absorption peaks at 330 nm (probe **A**) and 341 nm (probe **B**). Compared with the values obtained from other DFT methods (SVWN, PW91)⁴⁰, these values well match the experimental data. For the acidic environment, when probes **A** and **B** are protonated leading to opening of the spirolactam moieties, the SVWN functional⁴⁰ results in absorption peaks at 634 nm (probe **A**) and 639 nm (probe **B**). These values match better with our experimental data when compared with the values obtained from other DFT functional (B3LYP, PW91).⁴⁰ Thus, we can conclude that a correct choice of exchange-correlation functional is imperative to obtain the optical spectra of the fluorescent probes examined at different pH levels.

Probe photostability.

The photostability of the probes was evaluated by measuring fluorescence intensity every 10 min through continuous excitation of the probes under 640 nm or 800 nm for 5-min periods. Probe **A** exhibited a Stokes-shift fluorescence decrease of less than 1.0% under a 3-hour session excitation at 640 nm (Figure S19) while the Stokes-shift fluorescence intensity of probe **B** decreased by 3.0% under similar conditions (Figure S19). Probes **A** and **B** show better photostability than a commercially available near-infrared cyanine dye (IR-780) (Figure S19). Interestingly, using the lower energy one-photon excitation effectively prevents probe photo bleaching issues. Anti-Stokes shift fluorescence intensities of probes **A** and **B** show less than 0.7% and 2% decrease respectively during an identical 3-hour period of excitation at 800 nm (Figure S20).

Selectivity Experiments.

Probe selectivities were investigated at pH 3.2 and 7.4 in buffer solutions either with or without metal ions. The presence of 50 μM metal ions including Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Al^{3+} , Cd^{2+} , Mn^{2+} , Ni^{2+} , Cu^{2+} , Cr^{3+} , Fe^{3+} , and Ag^+ ions do not result in significant fluorescence change at pH 3.2 (Figure S23), demonstrating that probe response is unaffected by these cation metal ions. The effect of anions on the probes was also determined with 50 μM of various anions including Cl^- , I^- , Br^- , NO_3^- , NO_2^- , SO_4^{2-} , SO_3^{2-} , S^{2-} , HCO_2^{3-} , PO_4^{3-} or CO_3^{2-} ions in pH 3.2 or 7.4 buffer. These results show that probe response is also unaffected by the presence of any of these anions under excitation at 645 nm at pH 3.2 (Figure S24). Additionally, the probes exhibit sustained emission responses at pH 3.2 with 50 μM biothiols and amino acids such as alanine, arginine, cystine, cysteine, glycine, leucine, methionine, proline, tyrosine, and GSH (reduced glutathione) (Figure S25). Therefore, probes **A** and **B** can be used to explore pH-related biological processes without any significant interference from cations, anions, biothiols and amino acids in the biological samples.

Probe cytotoxicity.

We employed an MTS assay to study the probe cytotoxicity through incubation of the cells with the probes under concentration ranges from 5 to 20 μM for 48 h. Cell viability of 88.3% and 92.0% for probes **A** and **B** at 10 μM , respectively, convincingly demonstrated that the probes possess low cytotoxicity to the cells under these experimental conditions (Figure S26). The mannose residue of probe **B** can not only considerably enhance the probe

solubility in aqueous solutions but also improve the probe biocompatibility as probe **B** exhibited lower cytotoxicity.

Probe cellular imaging of lysosomes

Membrane-bound acidic Lysosomes maintain an acidic pH ranging from 4.5 to 5.0 allowing a wide variety of hydrolytic enzymes to function and break down proteins, nucleic acids, polysaccharides.⁴⁴ It is known that small molecules under 1 KDa easily diffuse through lysosomal membranes due to osmotic pressure.⁴⁴ Fluorescent probes bearing weakly basic lipophilic amines such as morpholine residues are easily trapped inside lysosomes due to the impermeability of the membrane as the amino groups become positively charged through protonation under lysosomal acidic pH conditions.⁴⁴ Probes **A** and **B** with pK_a values of 4.60 and 4.40 respectively are suitable for monitoring lysosomal pH.⁴⁴ We investigated whether probes **A** and **B** could specifically target lysosomes in live cells by using LysoTracker Green (a well-characterized lysosome specific probe) and conducting co-localization analyses (Figure 6). To validate the co-localization of probes **A** and **B** to lysosomes, we incubated LysoTracker Green and new probes with HeLa cells and collected images of the same observation field under different excitation and emission wavelengths to accomplish simultaneous visualization of LysoTracker Green and the probes in the same lysosomes.⁴⁴ The probes become fluorescent inside HeLa cells as lysosomal acidic pH activates fluorescence by opening the probe spirolactam ring structures. Probe **B** showed a stronger fluorescence signal than probe **A** under the same concentration as the mannose residue from probe **B** may be facilitating probe cellular uptake (Figure 6). The sections in the cells labelled positively by the LysoTracker Green were almost perfectly matched with those stained with probe **A** or **B** and the fluorescence microscopy co-localization analysis gave the Pearson's coefficients of 0.85 or higher between LysoTracker Green and the probes in live cells. These results demonstrate that probes **A** and **B** stay in the same lysosomes with LysoTracker green (Figure 6).

Probe near-infrared fluorescence visualization of lysosomal pH changes.

To investigate whether the probes can detect lysosomal pH changes, we conducted the cellular fluorescence imaging of lysosomal pH changes by incubating 10 μ M of the probes with HeLa and KB cells, and then putting the cells in buffers with pH from 2.5, 3.5, 4.5, 5.5, 6.5 to 7.5 holding 5 mg/L nigericin. The nigericin ionophore functions to homologize the intracellular pH and buffer pH.^{25, 30, 37, 45} Stokes-shift fluorescence images were first examined using the lamp-based Olympus IX-71 inverted microscope, and single-photon anti-Stokes-shift fluorescence was also examined using the same microscope with an external continuous 808 nm wavelength laser (Figure 7). Both probes **A** and **B** display very weak cellular Stokes-shift and anti-Stokes-shift fluorescence in the medium with physiological pH (7.4). Upon gradual lysosomal pH decrease from 7.5 to 3.5, gradual increases in cellular fluorescence intensities of the probes were observed in both Stokes-shift and anti-Stokes-shift fluorescence (Figures 7) as low pH levels breaks apart the spirolactam rings improving molecular conjugation. These cellular imaging results agree with how the probes respond to pH variance in buffers (Figures 2 and 3). We further used the probes to investigate pH changes in lysosomes with high-resolution using a Zeiss LSM510 confocal

microscope (Figures 8, 10, S41-S66). Probes **A** and **B** exhibit similar turn-on cellular fluorescence responses in both Stokes-shift and anti-Stokes-shift fluorescence to pH changes in lysosomes of live HeLa and KB cells (Figures 8–11), which is also consistent with anti-Stokes-shift emission responses to pH variance in buffers (Figures 2 and 3). Cellular anti-Stokes-shift fluorescence can still be observed at pH 6.5 in HeLa and KB cells (Figures 8 and 10). Merged images of Stokes-shift and anti-Stokes-shift fluorescence images change colors from bright yellow to weak green with pH variance from 2.5 to 7.5 (Figures 8 and 9), indicating that probes **A** and **B** can also offer fluorescence visualization of lysosomal pH variance with a double-checked capability through two near-infrared fluorescence channels. In addition, we conducted Stokes-shift fluorescence imaging of lysosomal pH variances in HUVEC-C and MDA-MB231 breast cancer cells employing an inverted fluorescence microscope (AMF-4306, EVOSfl, AMG) and proved that the probes could sensitively respond to pH variance in lysosomes in live cells (Figures S27-S38). These combined results persuasively suggest that probes **A** and **B** can be used to sensitively monitor lysosomal pH changes.

4. CONCLUSION

In summary, we synthesized two new near-infrared fluorescent probes capable of exhibiting Stoke-shift and one-photon anti-Stokes-shift fluorescence. These probes also detected pH changes in live cell lysosomes. The morpholine residue on the probes specifically binds to lysosomes and the mannose group in probe **B** improves biocompatibility and aqueous solubility. Both probes reacted measurably to pH variance in both buffers and lysosomes with significant increases of both Stoke-shift and one-photon anti-Stokes-shift fluorescence. They possess excellent photostability with low photo bleaching and high pH selectivity. The probe platforms enable the construction of a variety of fluorescent probes with spirolactam on-off switches for sensitive sensing of cellular metal ions, ATP, and reactive species involving oxygen, nitrogen and sulfur atoms through two Stoke-shift and one-photon anti-Stokes-shift fluorescence channels with a double-checked capability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Absorption spectra for probes **A** and **B** (10 μ M) in citrate-phosphate buffers with 1% ethanol.

Figure 2.
Stoke-shift emission spectra of 10 μ M probes **A** and **B** in citrate-phosphate buffers in the presence of 1% ethanol under excitation at 645 nm and 640 nm, respectively.

Figure 3.

Anti-Stokes-shift emission spectra of 10 μ M probes **A** and **B** in citrate-phosphate buffers in the presence of 1% ethanol with one-photon 800 nm excitation.

Figure 4.

Optimized structures of the probes of different configurations in neutral (water) and acidic (formic acid) solvent. (a) & (b) represent probes **A** and **B** with closed spirolactam moiety (neutral pH) respectively, (c) & (d) stand for protonated probes **A** and **B** with opened spirolactam moiety (acidic environment); perchlorate ion (ClO_4^-) is taken as a counter ion.

Figure 5.

UV-VIS spectra showing the absorption of different fluorescent probes calculated using TD-DFT with three different exchange correlation functionals. (a) & (b) the probes with closed spirolactam ring (neutral pH) respectively, (c) & (d) the protonated probes with open spirolactam ring (acidic pH), respectively.

Figure 6.

Images of HeLa cells together with 20 μM and 50 μM fluorescent probes **A** and **B**, 5 μM Lysotracker Green and Hoechst stains for 2 h in cell culture media. Confocal fluorescence microscope (Olympus IX 81) at 60 $\times$ magnification was employed to collect the images. Blue fluorescence between 425 and 475 nm of Hoechst was obtained under 405 nm excitation, and green fluorescence from 525 to 575 nm of Lysosensor green was acquired for under 488 nm excitation while near-infrared fluorescence from 650 to 700 nm of probe **A** or **B** was collected under 630 nm excitation.

Figure 7.

Stokes-shift and anti-Stokes-shift luminescence images, and their merged images at 40× magnification of HeLa cells together with 10 μ M of probes **A** and **B** in buffers with pH from 3.5, 4.5, 5.5, 6.5 to 7.5 together with 5 mg/L nigericin. Stokes-shift luminescence images were obtained with a band-pass filter Ex608–648/Em672–712 nm while anti-Stokes-shift luminescence images were measured from 672 to 712 nm under excitation at 808 nm with a CW laser. Note that dashed line circle in anti-Stokes-shift luminescence images indicates the

808 nm laser light spot. Images were performed by the lamp-based Olympus IX-71 inverted fluorescence microscope with scale bars of 20 μm .

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Figure 8.

Stokes-shift and anti-Stokes-shift luminescence images with scale bars of 20 μm at 40× magnification of Hela cells together with 10 μM of probes **A** and **B** in pH buffers from 2.5, 3.5, 4.5, 5.5, 6.5 to 7.5 holding 5 mg/L nigericin. Stokes-shift luminescence image (red-color) was acquired under 633 nm excitation with HeNe continuous wave laser while anti-Stokes-shift luminescence image (green-color) was obtained under 800 nm excitation with a pulse laser. Both Stokes-shift and anti-Stokes-shift luminescence were collected from 650 to 700 nm by using Zeiss LSM510 confocal fluorescence microscope.

Figure 9. Cellular luminescence intensity of probes **A** and **B** versus pH obtained from cellular luminescence images in Figure 8 under 633 nm and 800 nm excitation.

Figure 10.

Stokes-shift and anti-Stokes-shift luminescence images with scale bars of 20 μm at 40 $\times$ magnification of KB cells together with 10 μM of probes **A** and **B** in pH buffers from 2.5, 3.5, 4.5, 5.5, 6.5 to 7.5 holding 5 mg/L nigericin. Stokes-shift luminescence image (red-color) was acquired under 633 nm excitation with a HeNe continuous wave laser while anti-Stokes-shift luminescence image (green-color) was obtained under 800 nm excitation with a pulse laser. Both Stokes-shift and anti-Stokes-shift luminescence were collected from 650 to 700 nm by Zeiss LSM510 confocal fluorescence microscope.

Figure 11. Cellular luminescence intensity of probes **A** and **B** versus pH obtained from cellular luminescence images in Figure 10 under 633 nm and 800 nm excitation.

Scheme 1.

Stokes-shift and one-photon anti-Stokes-shift fluorescence mechanism.

Scheme 2.
Probe structural responses to pH changes

Scheme 3.
Synthetic approach to the probes.