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Fluorescent Probes for Sensitive and Selective Detection of pH Changes in Live Cells in Visible and Near-infrared Channels

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Abstract

We report five fluorescent probes based on coumarin-hybridized fluorescent dyes with spirolactam ring structures (A-E) to detect pH changes in live cell by monitoring visible and near-infrared fluorescence changes. Under physiological or basic conditions, the fluorescent probes A, B, C, D and E preserve their spirolactam ring-closed forms and only display fluorescent peaks in the visible region corresponding to coumarin moieties at 497, 483, 498, 497 and 482 nm, respectively. However, at acidic pH, the rings of the spirolactam forms of the fluorescent probes A, B, C, D and E open up, generating new near-infrared fluorescence peaks at 711, 696, 707, 715, and 697 nm, respectively, through significantly extended π -conjugation to coumarin moieties of the fluorophores. The fluorescent probes B and E can be applied to visualize pH changes by monitoring visible as well as near-infrared fluorescence changes. This helps avoid fluorescence imaging blind spots at neutral or basic pH, which typical pH fluorescent probes encounter. The probes exhibit high sensitivity to pH changes, excellent photostability, low auto-fluorescence background and good cell membrane permeability.

Graphical abstract

Five fluorescent probes bearing coumarin moieties with spirolactam ring structures have been developed to detect pH changes in visible and near-infrared channels.

1. Introduction

Intracellular pH serves very important roles in many processes of the cell functions and regulations, proliferation, phagocytosis, vesicle trafficking, cellular metabolism, enzymatic activity and cell apoptosis.^{1–4} Different compartments inside the cell have different intracellular pH values. For example, the pH value of lysosome in a typical mammalian cell is 4.7–6.0 while pH in mitochondria is around 8.0. Acidic intra-compartmental pH in organelles leads to activation of enzymes or denaturation of protein that are inactive in neutral conditions. There is a decrease in the intracellular pH (acidosis) in many neurologic diseases such as epilepsy, Alzheimer's disease, and Parkinson's disease^{5–7} as well as cancer.⁷ Therefore, the accurate monitoring of pH fluctuations in live cells are of significant importance to understand physiological and pathological processes, and also help explore cellular functions. Fluorescent probes for pH possess unique cellular imaging merits including excellent spatial and temporal resolution, easy operation, high sensitivity, and high signal-to-noise ratio.^{8–25} As a result, this powerful technique has attracted significant attention of scientists for monitoring the intracellular pH in live cells.^{26–28} Even though, large numbers of fluorescent probes have been developed,²⁹ only a few of the probes have been used for intracellular lysosomal pH monitoring and imaging in near-infrared region.^{30–37} Advantage of near-infrared fluorescence emission includes deep tissue light penetrations, optical transparency and low autofluorescence interference from biological tissues and samples.^{22, 24, 30–36} Furthermore, most commercial and reported fluorescent probes for detection of pH changes are based on monitoring single emission either in visible region or near-infrared region.^{8–25, 30–36} In order to address this fluorescence imaging blind spot issue in neutral or basic pH, we report fluorescent probes (**A–E**) based on coumarin-hybridized dyes bearing spirolactam ring structures with two excitation and emission wavelengths (visible as well as near-infrared region) to detect pH changes in live cells by monitoring fluorescence changes. Under physiological or basic conditions, the fluorescent probes **A**, **B**, **C**, **D** and **E** preserve the closed spirolactam forms, and only display moderate fluorescent peaks in the visible region corresponding to coumarin moieties at 497, 483, 498, 497 and 482 nm, respectively. However, the acidic pH effectively activates ring opening of the probe spirolactam forms, resulting in new near-infrared fluorescence peaks at 711, 696,

707, 715, and 697 nm, respectively (Scheme 1). The fluorescent probe **B** and **E** has been suitably used to visualize pH changes in live cells by monitoring visible and near-infrared changes in two fluorescence channels, and to avoid fluorescence imaging blind spots in basic condition, which commonly occur in most fluorescent probes for detection of pH in live cells. The fluorescent probes **B** and **E** show high sensitivity and good selectivity to pH over other metal ions, excellent photostability, low auto-fluorescence interference from biological samples and good cell membrane penetrability.

1. Materials and Methods

2.1 Instrumentation

400 MHz Varian Unity Inova NMR spectrophotometer instrument was used to obtain ^1H NMR and ^{13}C NMR spectra in CDCl_3 and DMSO-d_6 solutions. Chemical shifts (δ) are set in ppm relative to solvent residual peaks (^1H : δ 7.26 for CDCl_3 , δ 2.50 for DMSO-d_6 ; ^{13}C : δ 77.3 for CDCl_3) as internal standards. High-resolution mass spectrometer data (HRMS) were measured with fast atom bombardment (FAB) ionization mass spectrometer, double focusing magnetic mass spectrometer or matrix assisted laser desorption/ionization time of flight mass spectrometer. Absorption and fluorescence spectra were obtained by using Perkin Elmer Lambda 35 UV/VIS spectrometer and Jobin Yvon Fluoromax-4 spectrofluorometer, respectively.

2.2 Materials

Unless specifically indicated, all reagents and solvents were bought from commercial suppliers and used without further purification. Compound **9** was prepared and characterized according to our reported procedure.³⁸

Compound 3—When compound **2** (425 mg, 1.5 mmol), 20 mL of concentrated sulfuric acid (20 mL) and compound **1** (469 mg 1.5 mmol) were added to a 50-mL round-bottom flask, the reaction mixture was stirred at 100°C for 12 hours. After the mixture was cooled down to room temperature, the solution was poured into ice water. 70% perchloric acid (10 mL) was added to precipitate the product. After the crude product was filtered and dissolved in DCM (20 mL), the mixture was washed with water and dried over anhydrous sodium sulfate. The crude product was collected by filtrating and concentrating under reduced pressure. The residue was then purified by silica gel chromatography, using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (20:1, v/v) as eluent to obtain compound **3** as a green solid (500 mg, 60% yield). ^1H NMR (400 MHz, DMSO-d_6) δ 8.74 (s, 1H), 8.05 (d, J = 7.6 Hz, 1H), 7.80-7.76 (m, 1H), 7.72-7.68 (m, 1H), 7.43 (d, J = 7.6 Hz, 1H), 7.14 (d, J = 7.6 Hz, 1H), 6.99-6.92 (m, 4H), 3.52-3.50 (m, 4H), 3.38 (s, 4H), 2.70-2.69 (m, 4H), 1.87-1.86 (m, 4H), 1.17-1.14 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.1, 158.9, 152.8, 144.2, 134.2, 130.9, 129.5, 129.4, 128.9, 128.8, 128.5, 126.0, 121.4, 109.8, 105.8, 97.0, 50.8, 50.2, 45.4, 27.3, 21.0, 20.0, 13.1. HRMS (ESI): calculated for $\text{C}_{35}\text{H}_{33}\text{N}_2\text{O}_5^+$ [M-ClO_4] $^+$, 561.2384; found, 561.2368.

Compound 6—Compound **6** was prepared similarly by using compounds **1** and **5** according to the procedure to synthesize compound **3** (620 mg, 68% yield). ^1H NMR (400 MHz, DMSO-d_6) δ 8.85 (s, 1H), 8.05 (d, J = 8.0 Hz, 1H), 7.80-7.76 (m, 1H), 7.72-7.68 (m,

1H), 7.64-7.62 (d, $J = 9.2$ Hz, 1H), 7.43 (d, $J = 7.6$ Hz, 1H), 6.98 (s, 1H), 6.90-6.84 (m, 4H), 6.59 (s, 1H), 3.51-3.47 (m, 8H), 1.17-1.10 (m, 12H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 168.2, 158.8, 157.9, 156.6, 154.1, 153.4, 144.7, 134.3, 132.7, 130.9, 129.5, 129.4, 121.4, 128.2, 114.9, 111.7, 109.5, 107.4, 97.1, 96.9, 45.5, 45.4, 13.1. HRMS (ESI): calculated for $\text{C}_{33}\text{H}_{33}\text{N}_2\text{O}_5^+ [\text{M}-\text{ClO}_4]^+$, 537.2384; found, 537.2371.

Compound 8—Compound **8** was synthesized similarly by using compounds **5** and **7** according to the procedure to prepare compound **3** (410 mg, 55% yield). ^1H NMR (400 MHz, DMSO- d_6) δ 8.65 (s, 1H), 8.05 (d, $J = 7.2$ Hz, 1H), 7.78-7.70 (m, 2H), 7.50 (s, 1H), 7.43 (d, $J = 7.2$ Hz, 1H), 7.16 (s, 1H), 6.96-6.90 (m, 3H), 3.50-3.34 (m, 8H), 2.65 (s, 4H), 1.84 (s, 4H), 1.14 (s, 6H); ^{13}C NMR (100 MHz, DMSO- d_6) δ 167.6, 158.7, 158.1, 155.0, 153.2, 153.1, 151.6, 144.8, 133.4, 131.1, 130.1, 129.7, 129.1, 122.1, 117.0, 115.2, 110.5, 106.0, 103.8, 96.7, 55.6, 51.1, 50.5, 45.9, 27.3, 20.9, 19.9, 13.1. HRMS (ESI): calculated for $\text{C}_{35}\text{H}_{33}\text{N}_2\text{O}_5^+ [\text{M}-\text{ClO}_4]^+$, 561.2384; found, 561.2365.

Fluorescent probe A—Compound **3** (0.5 g, 0.76 mmol), N,N' -dicyclohexylcarbodiimide (0.17 g, 0.83 mmol) and 4-dimethylaminopyridine (0.11 g, 0.91 mmol) were dissolved in dichloromethane (15 mL). After the reaction mixture was stirred for 1 h at room temperature, 11-azido-3,6,9-trioxaundecan-1-amine (0.498 g, 2.28 mmol) in dichloromethane (5 mL) was added, and the reaction mixture was stirred overnight at room temperature. When 30 mL of dichloromethane was added to the reaction flask, the reaction mixture was washed with water and brine. The organic layer was collected, dried over anhydrous sodium sulfate, and filtrated. The filtrate was concentrated under a reduced pressure to get crude product. The crude product was purified by silica gel chromatography via dichloromethane/methanol (15/1, v/v) as eluent to obtain probe **A** as a light yellow solid (0.289 g, 49.8% yield). ^1H NMR (400 MHz, CDCl_3) δ 8.21 (s, 1H), 7.80 (d, $J = 6.8$ Hz, 1H), 7.46-7.38 (m, 2H), 7.19 (d, $J = 7.6$ Hz, 1H), 7.04 (s, 1H), 6.38-6.34 (m, 2H), 6.28-6.25 (m, 1H), 6.18 (s, 1H), 3.68-3.58 (m, 8H), 3.55-3.50 (m, 4H), 3.35-3.24 (m, 10H), 2.85-2.82 (m, 2H), 2.78-2.75 (m, 2H), 1.95-1.94 (m, 6H), 1.17-1.13 (m, 6H); ^{13}C NMR (100 MHz, CDCl_3) δ 168.1, 159.7, 157.0, 153.2, 153.1, 151.4, 148.9, 146.7, 139.9, 132.3, 131.0, 128.5, 128.4, 126.1, 124.1, 123.1, 119.1, 111.2, 109.1, 108.4, 106.1, 105.4, 101.8, 98.0, 70.8, 70.7, 70.6, 70.4, 70.1, 68.4, 63.7, 50.9, 50.3, 49.9, 44.5, 39.4, 27.7, 21.6, 20.7, 20.4, 12.8. HRMS (ESI): calculated for $\text{C}_{43}\text{H}_{48}\text{N}_6\text{O}_7 [\text{M}+\text{H}]^+$, 761.3657; found, 761.3654.

Fluorescent probe B—Probe **B** was synthesized by using compounds **4** and **6** through the same procedure to prepare probe **A** (light green color, 0.366g, 54% yield). ^1H NMR (400 MHz, CDCl_3) δ 8.28 (s, 1H), 7.83 (d, $J = 8.8$ Hz, 1H), 7.44-7.39 (m, 3H), 7.20-7.18 (m, 1H), 6.63 (dd, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz, 1H), 6.48 (d, $J = 2.4$ Hz, 1H), 6.39-6.37 (m, 2H), 6.29 (dd, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz, 1H), 6.19 (s, 1H), 3.60-3.49 (m, 14H), 3.44-3.38 (m, 6H), 3.36-3.30 (m, 6H), 1.23-1.14 (m, 12H); ^{13}C NMR (100 MHz, CDCl_3) δ 168.2, 159.5, 156.4, 153.1, 153.0, 151.5, 148.9, 146.5, 139.7, 132.4, 130.9, 130.0, 128.5, 128.4, 124.2, 123.1, 112.1, 111.2, 109.6, 109.2, 108.4, 105.2, 102.2, 98.0, 96.9, 70.7, 70.7, 70.6, 70.3, 70.1, 68.6, 63.7, 50.8, 45.1, 39.5, 25.8, 12.8, 12.7. HRMS (ESI): calculated for $\text{C}_{41}\text{H}_{48}\text{N}_6\text{O}_7 [\text{M}+\text{H}]^+$, 737.3657; found, 737.3654.

Fluorescent probe C—Probe C was prepared by using compounds **4** and **8** in the same way to synthesize probe A (light yellow color, 0.272g, 46% yield). ^1H NMR (400 MHz, CDCl_3) δ 8.18 (s, 1H), 7.83 (d, J = 8.0 Hz, 1H), 7.44-7.39 (m, 3H), 7.20-7.18 (m, 1H), 6.62-6.60 (m, 1H), 6.48-6.47 (d, J = 2.0 Hz, 1H), 6.18 (s, 1H), 5.97 (s, 1H), 3.60-3.53 (m, 12H), 3.45-3.39 (m, 4H), 3.33-3.31 (m, 4H), 3.16-3.07 (m, 4H), 2.96-2.93 (m, 2H), 2.47-2.44 (m, 2H), 2.08-2.05 (m, 2H), 1.86-1.83 (m, 2H), 1.23-1.19 (m, 6H); ^{13}C NMR (100 MHz, CDCl_3) δ 168.1, 159.7, 156.9, 153.2, 153.0, 151.4, 148.8, 146.7, 139.8, 132.3, 131.0, 128.5, 128.4, 126.1, 124.1, 123.1, 119.0, 111.3, 109.1, 108.4, 106.1, 105.4, 101.8, 98.0, 70.8, 70.7, 70.6, 70.4, 70.2, 70.1, 68.4, 63.7, 50.9, 50.3, 49.9, 44.5, 39.4, 29.9, 27.7, 21.6, 20.7, 20.4, 12.8. HRMS (ESI): calculated for $\text{C}_{43}\text{H}_{48}\text{N}_6\text{O}_7$ $[\text{M}+\text{H}]^+$, 761.3657; found, 761.3655.

Fluorescent probe D—Fluorescent probe A (0.220 g, 0.3 mmol), copper(I) iodide (0.067 g, 0.35 mmol) and triethylamine (0.1 mL, 0.91 mmol) were dissolved in dichloromethane (10 mL). After the reaction mixture was stirred for 10 min at room temperature, compound **9** (0.105 g, 0.45 mmol) in dichloromethane (5 mL) was added, and the reaction mixture was stirred overnight at room temperature. When 30 mL of dichloromethane was added to the flask, the reaction mixture was washed with water and brine three times. The organic layer was collected, dried over anhydrous sodium sulfate, and filtrated. The filtrate was concentrated under a reduced pressure to get crude product. The crude product was purified by silica gel chromatography via ethyl acetate/methanol (20/1, v/v) as eluent to obtain probe **D** as a light yellow solid (0.216 g, 75% yield). ^1H NMR (400 MHz, CDCl_3) δ 8.16 (s, 1H), 7.75-7.72 (m, 2H), 7.37-7.33 (m, 2H), 7.12 (d, J = 7.6 Hz, 1H), 6.98 (s, 1H), 6.33-6.29 (m, 2H), 6.22-6.20 (m, 1H), 6.11 (s, 1H), 4.58 (s, 2H), 4.42-4.40 (m, 2H), 3.73-3.71 (m, 2H), 3.63-3.50 (m, 18H), 3.45-3.42 (m, 8H), 3.27-3.20 (m, 10H), 2.78-2.69 (m, 4H), 1.89-1.88 (m, 4H), 1.11-1.07 (m, 6H); ^{13}C NMR (100 MHz, CDCl_3) δ 168.1, 159.8, 153.1, 153.0, 151.4, 148.8, 146.9, 146.8, 144.7, 140.0, 132.3, 130.8, 128.4, 128.3, 126.1, 124.3, 124.1, 122.9, 119.2, 110.7, 109.1, 108.2, 105.9, 105.1, 101.5, 97.9, 72.7, 72.6, 70.6, 70.5, 70.4, 70.3, 70.2, 69.6, 69.5, 68.4, 64.6, 63.8, 61.5, 50.4, 50.3, 49.8, 44.5, 39.4, 27.7, 21.5, 20.6, 20.4, 12.8. HRMS (ESI): calculated for $\text{C}_{54}\text{H}_{68}\text{N}_6\text{NaO}_{12}$ $[\text{M}+\text{Na}]^+$, 1015.4787; found, 1015.4796.

Fluorescent probe E—Probe E was synthesized by the same procedure to prepare probe **D** using compounds **9** and probe **B** (light green color, 0.166g, 70% yield). ^1H NMR (400 MHz, CDCl_3) δ 8.28 (s, 1H), 7.79 (d, J = 8.0 Hz, 2H), 7.45-7.39 (m, 3H), 7.18 (d, J = 6.8 Hz, 1H), 6.63-6.60 (m, 1H), 6.46 (s, 1H), 6.39-6.35 (m, 2H), 6.29-6.25 (m, 1H), 6.17 (s, 1H), 4.65 (s, 2H), 4.48-4.45 (m, 2H), 4.25 (s, 1H), 3.79-3.77 (m, 2H), 3.65-3.57 (m, 22H), 3.53-3.47 (m, 6H), 3.45-3.31 (m, 8H), 1.22-1.13 (m, 12H); ^{13}C NMR (100 MHz, CDCl_3) δ 168.2, 159.5, 156.4, 153.1, 153.0, 151.5, 148.9, 146.5, 144.6, 139.8, 132.5, 130.9, 130.1, 128.5, 124.3, 124.2, 123.0, 112.0, 109.6, 109.2, 108.4, 105.1, 102.2, 98.0, 96.9, 75.6, 72.7, 72.4, 70.7, 70.7, 70.6, 70.5, 70.4, 70.3, 70.3, 70.0, 69.5, 68.5, 64.6, 63.7, 61.8, 61.3, 59.1, 50.4, 45.1, 44.5, 39.5, 33.1, 24.6, 12.8, 12.7. HRMS (ESI): calculated for $\text{C}_{52}\text{H}_{68}\text{N}_6\text{NaO}_{12}$ $[\text{M}+\text{Na}]^+$, 991.4787; found, 991.4794.

Cell culture and fluorescence imaging: Breast cancer cells (MDA-MB231) and HUVEC-C cells obtained from ATCC were cultured according to the reported protocols.³⁹ Cells were plated at a density of 1×10^5 cells/well on 12 well culture plates and incubated overnight at 37 °C in a 5% CO₂ incubator. After 24 h incubation, the media was removed and cells were rinsed twice with $1 \times$ PBS, and then added serum free media to starve the cells for 2 hours. After that, the cells were further incubated in fresh serum free media containing 10 μ M of probe **A**, **B**, **C**, **D** or **E**, and 2 μ M Lysosensor blue DND-167 for 1 hour at 37 °C. For Probe **B** experiments were carried out at 5 μ M concentration of dye as well. After an hour of incubation, the cells were rinsed three times with potassium rich PBS at different pH values of 4.5, 5.5, 6.5, 7.5 or 8.5. The cells were incubated further with nigericin (1 μ g/mL) for 5 minutes in respective potassium rich PBS buffer.^{40, 41} Fluorescence images were acquired at 60 $\times$ magnification using an inverted fluorescence microscope (AMF-4306, EVOSfl, AMG).

MTS assay: MDA-MB231 cells obtained from ATCC, were cultured according to previous protocols.³⁶ Cells were plated on a 96-well culture plate at a density of 2×10^4 cells per well and incubated at 37 °C in a 5% CO₂ incubator for overnight. Fresh media with different concentrations (0, 5, 10, 15, 20 or 50 μ M) of probe **A**, **B**, **C**, **D** or **E** were added to the wells. Each probe concentration was measured in 6 replicates. Blanks were prepared at different concentrations (0, 5, 10, 15, 20 or 50 μ M) of probe **A**, **B**, **C**, **D** or **E** but without cells. The plates were incubated at 37 °C in a 5 % CO₂ incubator for 48 h. To these cells MTS reagent was added and further incubated for 4 h followed by absorbance measurement at 490 nm.²⁴

Computational Methods: The structures of probes **A**, **B** and **C** in basic and acidic conditions, were built using the GaussView 5.0.9 program.⁴² Geometry optimizations of the six structures of probes **A**, **B** and **C** were performed, using Density Functional Theory (DFT) with B3LYP functional^{43, 44} and the 6-31G(d) basis set in Polarizable Continuum Model (PCM)^{43,44} with a dielectric constant of 78.4 for representing the water solvent. The resulting HOMO and LUMO orbitals were visualized using GaussView⁴¹.

3. Results and Discussion

3.1. Synthetic approach

The synthetic approach to the fluorescent probes **A**, **B**, **C**, **D** and **E** for pH sensing is outlined in Scheme 2. Aldol condensation reaction was conducted in the presence of concentrated sulfuric acid, using (4-(diethylamino)-2-hydroxyphenyl)(2-carboxyphenyl)methanone (**1**) and Coumarin **334** (**2**) to yield a near-infrared fluorophore,⁴⁵ 4-(2-carboxyphenyl)-7-(diethylamino)-2-(11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinolin-10-yl)chromenylium (**3**). The fluorescent probe **A** was prepared by reacting compound **3** with 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-amine (**4**) through a condensation reaction of the carboxylic acid of the fluorophore (**3**) with an amine group of compound **4** to form a nonfluorescent spirocyclic lactam ring structure at pH 7.4. Compounds **6** and **8** were synthesized similar to that for the synthesis of compound **3**. Fluorescent probes **B** and **C** were synthesized according to the same procedure to prepare probe **A**. Use of azide residue through oligo(ethylene glycol) tethered spacer is expected to enhance hydrophilicity of the probes and allow for further functionalization of the probes if

needed with different hydrophilic residues such as carbohydrate and peptides through click chemistry. In order to demonstrate further functionalization of the probes via click chemistry, we prepared fluorescent probes **D** and **E** by modifying probes **A** and **B** with ethynyl-functionalized oligo(ethylene glycol) (**9**) via click chemistry in the presence of CuI (Scheme 2).

3.2. Absorption responses of fluorescent probes to pH

The absorption responses of the fluorescent probes to different pH values were investigated in citric phosphate buffer solutions. Under basic and neutral conditions in 5 mM citric phosphate buffer solutions containing 40% ethanol for probes **A**, **B** and **C**, and containing 20% ethanol for probes **D** and **E**, fluorescent probes **A**, **B**, **C**, **D** and **E** display strong absorptions peaks at 441 nm, 429 nm, 436 nm, 446 and 430 nm with molar absorptivity of 1.9×10^4 , 3.1×10^4 , 2.0×10^4 M⁻¹cm⁻¹, 2.1×10^4 M⁻¹cm⁻¹ and 2.1×10^4 M⁻¹cm⁻¹, respectively. These probes show extremely weak absorption peaks between 650 and 700 nm (Figure 1) because they maintain closed spirolactam ring structures and only show fluorescence of the coumarin moieties. However, as the pH decreases from pH 7.6 to 1.6, new absorption peaks at 675 nm, 661 nm, 678 nm, 679 and 662 nm corresponding to probes **A**, **B**, **C**, **D** and **E**, respectively, appear and intensify with increasing acidic strength of buffers. The appearance of the new peaks in near-infrared region under acidic conditions results from the opening of the spirolactam rings with significantly extended π -conjugation to the coumarin moieties of the probes. At pH 2.0, fluorescent probes **A**, **B**, **C**, **D** and **E** display molar absorptivity of 7.0×10^3 M⁻¹cm⁻¹ at 675 nm, 5.4×10^3 M⁻¹cm⁻¹ at 661 nm, 8.2×10^3 M⁻¹cm⁻¹ at 678 nm, 5.5×10^3 M⁻¹cm⁻¹ at 679 nm and 3.0×10^3 M⁻¹cm⁻¹ at 662 nm, respectively. In addition, absorption peaks at 441 nm, 425 nm, and 436 nm corresponding to probes **A**, **B**, and **C** also increase with enhanced acidic strengths, respectively (Figure 1). At pH 2.0, fluorescent probes **A**, **B**, **C**, **D** and **E** display molar absorptivity of 1.7×10^4 M⁻¹cm⁻¹ at 441 nm, 2.1×10^4 M⁻¹cm⁻¹ at 429 nm, 1.7×10^4 M⁻¹cm⁻¹ at 436 nm, 1.7×10^4 M⁻¹cm⁻¹ at 446 nm and 2.0×10^4 M⁻¹cm⁻¹ at 430 nm, respectively. Fluorescent probes **A**, **C** and **D** exhibit longer absorption peaks than the probes **B** and **E** due to their more rigid π -conjugated structures.

3.3. Fluorescence responses of fluorescent probes to pH

Effect of pH on fluorescence intensity of fluorescent probes was studied in citric phosphate buffer solutions. Under physiological or basic conditions, probes **A**, **B**, **C**, **D** and **E** keep their closed spirolactam ring structures and only show fluorescent peaks in the visible regions corresponding to coumarin moieties at 497, 483, 498, 497 and 482 nm, respectively. Changing the buffer pH from pH 7.6 to 1.6 results in new near-infrared fluorescence peaks of the probes **A**, **B**, **C**, **D** and **E** at 711, 696, 707 nm, 715 nm, and 697 nm, respectively, with increases in the fluorescence peak intensities (Figure 2). The appearance of the new near-infrared fluorescence peaks in acidic condition are due to ring-opening of the spirolactam forms with significantly enhanced π -conjugation of the probes. The Henderson–Hasselbalch-type mass action equation was employed to calculate the pK_{cycl} values of the probes **A**, **B**, **C**, **D** and **E** related to the spirolactam ring opening of the fluorophores, and obtained values of 3.0, 4.0, 3.2, 3.0 and 4.0 (Scheme 1), respectively. In addition, fluorescent

peaks of the probes **A**, **B** and **C** at 497 nm, 482 nm and 498 nm also increase with increasing acid strength (Figure 3). The probes **A**, **B**, **C**, **D** and **E** show excellent reversible fluorescent responses to pH from 2.0 to 8.0 and possess fluorescence quantum yields of 0.27%, 1.85%, 0.65%, 1.29% and 2.45% in pH 2.0 buffers at excitation wavelength of 625 nm, respectively. The probes **A**, **B**, and **C** show fluorescence quantum yields of 2.73%, 2.64%, 4.86% corresponding to fluorescence of the coumarin moieties in pH 2.0 buffers containing 40% ethanol while probes **D** and **E** display 4.74% and 3.34% in pH 2.0 buffers containing 20% ethanol at excitation wavelength of 425 nm, respectively. In addition, fluorescent probes **A**, **C** and **D** display slightly longer fluorescence peaks than the probes **B** and **E** because of their more rigid π -conjugation structures.

3.4 Computational Analysis

The structure of probe **B** in basic conditions exhibited a conformation in which oligo(ethylene glycol) azide moiety was found in a parallel position relative to its fluorophore moiety. Spirolactam ring opening of probe **B** in acidic conditions did not cause much significant structural change except enhanced π -conjugation of the fluorophore, this is likely because the oligo(ethylene glycol) azide moiety remained approximately parallel in relation to the fluorophore moiety (Figure 4). The HOMO orbitals of probes **B** and **C** in basic conditions mainly show contributions from the aromatic nitrogen atom of the fluorophore moiety, with some additional contribution from the phenyl ring positioned adjacent to the spirolactam bond (Figures 4 and S28). In the HOMO orbital of probe **A** in basic conditions, there is a major contribution from the coumarin moiety (Figure S27 in supporting information). The LUMO orbitals of all three of the probes in basic condition are found to be centered on the coumarin moiety (Figures 4, Figures S27 and S28). In acidic conditions, the HOMO orbitals of all the three probes are very similar and exhibit contributions from both the coumarin and the fluorophore moiety (Figure 4, Figures S27 and S28). The LUMO orbitals of the three probes in acidic conditions are also very similar, they show contribution distributed across the coumarin and fluorophore moieties, we also see some of the orbital found on the phenyl ring adjacent to the amide bond (Figure 4, Figures S27 and S28).

3.5 The selectivity of fluorescent probes to pH over metal ions

We investigated whether the presence of different metal ions could interfere with fluorescence response of the probes to pH. The results showed that fluorescent probes **A**, **B**, **C**, **E** and **D** display selective fluorescence responses to pH over metal ions such as Ca(II), Mg(II), Fe(II), Al(III), Fe(III), Mn(II), Cr(II), Cu(II), Co(II), Ag(I), Zn(II), Ni(II), Hg(II) and Cd(II) ions (Figure 5). In addition, the presence of 200 μ M anions such as Cl^- , I^- , Br^- , NO_2^- , NO_3^- , SO_3^{2-} , SO_4^{2-} , S^{2-} , HCO_3^- and CO_3^{2-} doesn't cause any significant response changes of probe **B** to pH 2.0 or 7.6 (Figure S25). The presence of 50 μ M amino acids such as L-alanine, DL-asparagine, DL-citrulline, DL-glutamic acid, DL-leucine, DL-cysteine, DL-methionine, glycine, DL-proline, DL-valine, DL-tyrosine, DL-threonine, L-cysteine and glutathione doesn't cause significant interference with fluorescence responses of the probe **B** to pH 2.0 or 7.6 (Figure S26).

3.6. Photostability of the fluorescent probes

We examined the photostability of the probes through continuous excitation of them with 5-min intervals and evaluation of fluorescence intensity with every 10 min. Probe **A** exhibited good photostability with its fluorescence decrease by 4.5% under 1-hour excitation and by 9.5% under 3-hour excitation at 690 nm (Figure 6). Fluorescent probes **B** and **C** displayed the almost same excellent photostability as the probe **A**, their fluorescence intensity decreased by 3.7% and 8.4% under one-hour excitation and by 2.7% and 7.8% under three-hour excitation, respectively (Figure 6). Probes **A-E** display similar photostability compared with that of cyanine dye (IR-780) (Figure 6).

3.7. Cytotoxicity of the fluorescent probes

MTS assay was carried out with MDA-MB231 cells to measure the effect of these fluorescent probes on cell viability (Figure 7). Probe **A** and **D** shows greater cell viability (>70%) compared to other probes at all concentrations (5, 10, 15, and 20 μ M) tested. In the presence of probes **B** and **C** the cell viability was greater than 70% at 5 and 10 μ M concentration. Probe **E** shows cell viability greater than 80% at 5, 10 and 15 μ M concentration. However, the cell viability decreases significantly when the probe concentration is greater than 10 μ M, with viability less than 50% at concentration of 50 μ M for all five probes (Figure 7). Introduction of longer oligo(ethylene glycol) via click chemistry to probes **A** and **B** reduces cytotoxicity slightly since cytotoxicity of probes **D** and **E** is a little lower than that of probes **A** and **B** (Figure 7).

3.8. Fluorescence imaging of pH in live cells

The ability of the probes to detect the intracellular pH changes was carried out by performing live cell imaging in MDA-MB231 and HUVEC-C cells. Based upon the MTS assay data, we chose to 10 μ M concentration of fluorescent probes to visualize the pH change in cells. As probe **B** and **E** showed higher sensitivity for cell staining compared to probes **A**, **C** and **D**, we also carried out imaging experiments for probes **B** and **E** at 5 μ M concentration. In addition, cell imaging with probe **B** was carried out in both cell lines. We incubated cells in media with different pH values ranging from 4.5 to 8.5 containing probes and lysosensor blue DND-167 in the presence of nigericin (1 μ g/mL), which was used to equilibrate the intracellular and extracellular pH. Probe **B** shows strong near-infrared fluorescence at acidic pH for both MDA-MB231 and HUVEC-C cells, and the fluorescence decreases with pH increase (Figures 8, 9, S31-S32); indicating that probe **B** is very sensitive to pH changes and can sense pH changes in live cells. The co-localization analysis based on the Pearson's coefficient shows a value of 0.85 or higher for probe **B** with the commercial LysoSensor Blue, indicating that the probe **B** and LysoSensor Blue stayed in the same cellular compartment (Figure S38). However, visible fluorescence intensity of probe **B** observed using GFP filter is not sensitive to pH changes as we still observed the visible fluorescence at pH 8.5. The enlarged images of probes **A**, **B**, and **C** shows the localization of probe and LysoSensor Blue in same cellular compartments (Figures S38-S41). This unique feature of the probe, i.e. emission wavelength is sensitive to pH change, can show location of the probe at basic pH values in live cells and help avoid blind fluorescence imaging spots, which are typically issues of fluorescent probes for detection of lysosomal pH in live cells.

Probe **E** also shows strong near-infrared fluorescence at acidic pH for MDA-MB231 cells, and the fluorescence decreases with pH increase (Figures 10, S37). Strong fluorescence signals for probes **A**, **C** and **D** were observed using GFP filter (Figures 11, S33-S36). However, no near-infrared fluorescence signals were observed in CY5 channel. Probes **A** and **C** display strong optical responses to pH changes in solution but show weak signal in live cells. This may be due to more hydrophobic structures of the probes **A** and **C** with low pK_{cycl} values related to the spirolactam ring opening of the fluorophores compared to the probes **B** and **E** (Scheme 1). We also carried out experiments in the presence and absence of nigericin at pH 4.5 and 8.5 (Figures S42-S44). Cells incubated in the presence of nigericin showed increased fluorescence compared to cells incubated without nigericin. This increased fluorescence observed is due to the balance of intracellular and extracellular pH with the addition of the ionophore nigericin.

4. Conclusion

We have successfully developed three fluorescent probes (**A**, **B**, **C**, **D** and **E**) with different hydrophobic structures for detection of pH changes in live cells in order to avoid blind fluorescence imaging spots at basic pH values. The fluorescent probes **A**, **C** and **D** possess more hydrophobic structures with lower pK_{cycl} constants related to the spirolactam ring opening, and display insensitive fluorescence responses to pH changes in visible fluorescence region and no near-infrared fluorescence was detected in live cells although they show sensitive fluorescence responses to pH changes in solution. However, the fluorescent probes **B** and **E** exhibit very sensitive near-infrared fluorescence responses to pH changes both in live cells and solution while their visible fluorescence can be used to locate the probe location in basic pH values and avoid blind fluorescence imaging spots under basic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Absorption spectra of 5 μM probes **A**, **B**, **C**, **D** and **E** in 5 mM citric phosphate buffers with different pH values containing 40% ethanol for probes **A**, **B** and **C**, and 20% ethanol for probes **D** and **E**.

Figure 2.

Fluorescence spectra of 5 μM probes **A**, **B**, **C**, **D** and **E** in 5 mM citric phosphate buffers with different pH values containing 40% ethanol for probes **A**, **B** and **C**, and 20% ethanol for probes **D** and **E** at excitation of 625 nm.

Figure 3.

Fluorescence spectra of 5 μ M probes **A**, **B**, **C**, **D** and **E** in 5 mM citric phosphate buffers with different pH values containing 40% ethanol for probes **A**, **B** and **C**, and 20% ethanol for probes **D** and **E** at excitation of 425 nm.

Figure 4.

The optimized structures of fluorescent probe **B** in basic and acidic conditions, with atoms colored as follows carbon is grey, hydrogen is white, nitrogen is blue and oxygen is red. Visualizations of their HOMO and LUMO orbitals are colored by wavefunction in either red or green.

Figure 5.

Fluorescence responses of probes 5 μM **A**, **B**, **C**, **D** and **E** to pH 7.6 and 2.0 over metal ions in the absence and presence (200 μM) metal ions in 5 mM citric phosphate buffer contains 40% ethanol for probes **A**, **B** and **C**, and 20% ethanol for probes **D** and **E** at excitation of 625 nm.

Figure 6. Relative fluorescence intensities of 5 μ M probes **A**, **B**, **C** and cyanine dye IR-780 in pH 4.4 buffer containing 40% ethanol, and 5 μ M probes **D**, **E** and cyanine dye IR-780 in pH 4.4 buffer containing 20% ethanol as a function of time in 3 hours under excitation at 625 nm.

Figure 7.

Cytotoxicity of probes **A**, **B**, **C**, **D** and **E** tested by MTS assay. The MDA-MB-231 cells were incubated in presence of 5, 10, 15, 20 or 50 μM of probes **A**, **B**, **C**, **D** or **E** for 48 h. Cell viability was measured by adding the MTS reagent and measuring the formation of formazan at 490 nm. The absorbance measured at 490 nm is directly proportional to the cell viability. The error bars indicate ± S.D.

Figure 8.

Fluorescence images of MDA-MB231 cells incubated with 10 μ M fluorescent probe **B**.

MDA-MB231 cells were incubated with 10 μ M probe **B** at different pH values ranging from pH 4.5 to 8.5 in presence of LysoSensor blue DND-167 (2 μ M) and nigericin (1 μ g/mL).

Figure 9.

Fluorescence images of HUVEC-C cells incubated with fluorescent probe **B**. HUVEC-C cells were incubated with 10 μM probe **B** at different pH values ranging from pH 4.5 to 8.5 in presence of LysoSensor blue DND-167 (2 μM) and nigericin (1 μg/mL).

Figure 10.

Fluorescence images of MDA-MB231 cells incubated with 10 μM fluorescent probe **E**. MDA-MB231 cells were incubated with 10 μM probe **E** at different pH values ranging from pH 4.5 to 8.5 in presence of LysoSensor blue DND-167 (2 μM) and nigericin (1 μg/mL).

Figure 11.

Fluorescence images of MDA-MB231 cells incubated with fluorescent probe **A**, **C** or **D**. MDA-MB231 cells were incubated with 10 μ M probe **A**, **C** or **D** at pH values pH 4.5 and 7.5 in presence of LysoSensor blue DND-167 (2 μ M) and nigericin (1 μ g/mL).

Scheme 1.

Chemical structure responses of fluorescent probes to pH changes in both visible and near-infrared fluorescence regions.

Scheme 2.

Synthetic approaches to prepare fluorescent probes A-E