ABSTRACT: Many organelles, such as lysosomes and mitochondria, maintain a pH that is different from the cytoplasmic pH. These pH differences have important functional ramifications for those organelles. Many cellular events depend upon a well-compartmentalized distribution of H+ ions spanning the membrane for the optimal function. Cells have developed a variety of mechanisms that enable the regulation of organelle pH. However, the measurement of organelar acidity/alkalinity in living cells has remained a challenge. Currently, most existing probes for the estimation of intracellular pH show a single -organelle targeting capacity. Such probes provide data that fails to comprehensively reveal the pathological and physiological roles and connections between mitochondria and lysosomes in different species. Mitochondrial and lysosomal functions are closely related and important for regulating cellular homeostasis. Accordingly, the design of a single fluorescent probe that can simultaneously target mitochondria and lysosomes is highly desirable, enabling a better understanding of the crosstalk between these organelles. We report the development of a novel fluorescent sensor, rhodamine−coumarin pH probe (RCPP), for detection of organellar acidity/alkalinity. RCPP simultaneously moves between mitochondrion and lysosome subcellular locations, facilitating the simultaneous monitoring of pH alterations in mitochondria and lysosomes.
lyosomal markers (e.g., LysoTracker) as fluorescent signals are both stable and highly selective.

Mitochondria and lysosomes work synchronously to execute and regulate metabolic processes; accordingly, the crosstalk between these two organelles is critical for maintaining cellular homeostasis in eukaryotes.29–32 Loss of this crosstalk, associated with abnormal H+ gradients, can result in apoptosis.31,35 Defective mitochondria–lysosome interactions are often associated with cardiovascular diseases,33 neurodegenerative diseases,9,34 and cancer.34 To gain further insight into the interactions between mitochondria and lysosomes, and the role of the H+ gradient in this process, we have sought to design a single fluorescent probe that can simultaneously target mitochondria and lysosomes and provide real-time intracellular pH information on these organelles.

**MATERIALS AND METHODS**

**Cell Culture and Live Cell Imaging.** HeLa cells were obtained from American Type Cell Culture Collection (ATCC). HeLa cells used in this study were maintained in the RPMI-1640 medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL penicillin, and streptomycin in a humid atmosphere containing 5% CO2 at 37 °C. Cells were plated on an imaging dish and the medium was replaced with phosphate-buffered saline (PBS) (37 °C) once the cells achieved 70–80% confluency. Cells were treated with nigericin (1 μM) and equilibrated for 5 min prior to obtaining images using previously published procedures. In an extracellular acidic pH culture system, cells were incubated simultaneously with RCPP (10 μM), 2 μM LysoTracker Deep Red (ThermoFisher, E6/E4: 647/668 nm), and Hoechst 3342 at 37 °C for 30 min followed by 2× washes with PBS and one wash with a full medium prior to confocal imaging analysis. In an extracellular alkaline pH culture system, cells were incubated simultaneously with RCPP (10 μM), 2 μM MitoTracker Deep Red FM (ThermoFisher, E6/E4: 644/665 nm), and Hoechst 3342 at 37 °C for 30 min, followed by 2× washes with PBS and one wash in the full medium prior to confocal imaging analysis. All pseudocolor image captures used Olympus standard excitation and emission filter sets (λex/λem: 405/419–465, 488/500–550, and 592.5/608–648 nm).

**Synthesis Procedure.** Reagents and solvents available from commercial sources were used as received unless otherwise noted. Thin-layer chromatography (TLC) was performed using Sigma-Aldrich TLC plates, silica gel 60F-254 over the glass support, and a 0.25 μm thickness. Flash column chromatography was performed using Alfa Aesar silica gel, with particle size of 230–400 mesh. Melting points were determined using a MELTEMP melting point apparatus and were uncorrected. 1H and 13C nuclear magnetic resonance (NMR) spectra were measured with a Varian UNITY INOVA instrument at 400 and 100 MHz, respectively. The chemical shifts (δ) were reported in reference to solvent residual peaks (dimethyl sulfoxide (DMSO)-d6). 1H NMR (400 MHz, DMSO-d6) δ ppm 6.73 (d, J = 2.2 Hz, 1H), 6.77 (dd, J = 8.4 Hz, J = 2.2 Hz), 7.44 (d, J = 8.5 Hz, 1H), and 7.56 (s, 1H). 13C NMR (100 MHz, DMSO-d6) δ ppm 158.0, 157.4, 156.0, 152.4, 130.2, 115.0, 114.7, 112.5, and 102.3. 

**Synthesis of Compound 2.** Compound 2 was prepared by following the previously published procedure of Wang et al. with minor modification.36 Briefly, a reaction mixture of 2,4-dihydroxy benzaldehyde (5.60 g, 40.5 mmol), N-acetylglycine (4.68 g, 40 mmol), and anhydrous sodium acetate (19.72 g, 120 mmol) in acetic acid (80 mL) was stirred under reflux for 8 h. The reaction mixture was poured onto ice to give a yellow precipitate, which was then directly subjected to vacuum filtration. The yellow solid was washed with ice water to afford the crude product of 6.86 g (26 mmol). The crude product was then refluxed in a solution of conc. HCl and EtOH (2:1, 60 mL) for 1 h, and then 80 mL of cold water was added to dilute the solution. The solution was then cooled in an ice bath and NaNO3 (2 equiv, 52.5 mmol, 3.63 g) was added portion by portion. The solution was vigorously stirred for 10 min, and then Na3 was added (2 equiv, 52.5 mmol, 3.41 g). After stirring for another 15 min, the reaction mixture was filtered, and the precipitate was washed with water to afford a brown solid (2 g, 49% yield). 1H NMR (400 MHz, DMSO-d6) δ ppm 6.73 (d, J = 2.2 Hz, 1H), 6.77 (dd, J = 8.4 Hz, J = 2.2 Hz), 7.44 (d, J = 8.5 Hz, 1H), and 7.56 (s, 1H). 13C NMR (100 MHz, DMSO-d6) δ ppm 158.0, 157.4, 156.0, 152.4, 130.2, 115.0, 114.7, 112.5, and 102.3.

**Synthesis of Compound 4.** Compound 3 was prepared by following our previously published procedure.36 A mixture of an azido-rodervative derivative (compound 3, 600 mg, 1 mmol) and 1, 4-diethynylbenzene (150.7 mg, 1.2 mmol), CuSO4 (6 mg, 0.05 mmol), and sodium l-ascorbate (19.8 mmol, 0.1 mmol) in t-BuOH/H2O (1:1, 4 mL) was stirred at room temperature for 12 h. After the solvent was removed under reduced pressure, the resulting residue was subjected to silica gel chromatography (dichloromethane/methanol 20:1) to give compound 4 (410 mg, 55% yield) as a colorless oil. 1H NMR (400 MHz, DMSO-d6) δ ppm 1.03 (t, J = 6.9 Hz, 12H), 4.18 (s, 2H), 4.21 (s, 1H), 4.63 (t, J = 6.1 Hz, 2H), 4.74 (t, J = 6.5 Hz, 2H), 6.12–6.20 (m, 4H), 6.27 (d, J = 2.2 Hz, 2H), 6.94–6.94 (m, 1H), 7.42 (s, 1H), 7.45–7.52 (m, 3H), 7.72 (d, J = 8.1 Hz, 2H), 7.75–7.78 (m, 1H), and 8.30 (s, 1H). 13C NMR (100 MHz, DMSO-d6) δ ppm 169.5, 151.7, 151.5, 149.7, 149.8, 147.8, 139.0, 132.6, 132.1, 130.5, 130.3, 130.1, 122.7, 127.5, 127.9, 114.2, 122.5, 97.5, 97.3, 124.8, 106.4, 128.7, 128.5, 124.8, 82.3, 81.4, 76.2, 45.2, 51.3, 47.0, and 12.8.

**Synthesis of RCPP.** A mixture of compound 4 (0.3 mmol, 220 mg), 3-azido-7-hydroxycoumarin (95.9 mg, 0.5 mmol), CuSO4 (10% mmol), and sodium l-ascorbate (20% mmol) in t-BuOH/H2O (1:1, 4 mL) was stirred at room temperature for 4 days. After the solvent was removed under reduced pressure, the resulting residue was subjected to silica gel chromatography (dichloromethane/methanol 20:1) to afford 30 mg of the target compound, RCPP (0.03 mmol, 10.8% yield, yellow solid). 1H NMR (400 MHz, DMSO-d6) δ ppm 0.97–1.06 (t, J = 6.99 Hz, 12H), 3.13–3.39 (m, 8H), 4.19 (s, 2H), 4.65 (t, J = 6.4 Hz, 1H), 4.76 (t, J = 5.2 Hz, 1H), 6.10–6.21 (m, 4H), 6.27 (s, 2H), 6.84 (s, 1H), 6.86–6.91 (m, 1H), 6.92–6.98 (m, 1H), 7.40–7.50 (m, 3H), 7.71–7.79 (m, 2H), 7.81 (d, J = 6.99 Hz, 2H), 7.97 (d, J = 6.07 Hz, 2H), 8.30 (s, 1H), 8.63 (s, 1H), and 9.01 (s, 1H). 13C NMR (100 MHz, DMSO-d6) δ ppm 165.9, 165.0, 130.3, 130.4, 130.2, 128.7,
RESULTS AND DISCUSSION

Design Rationale of the RCPP Probe. We and others have previously developed a series of lysosome-targeting fluorescent probes for the study of lysosomal activity. In our present study, two fluorophores (rhodamine and coumarin) were selected to construct a broad pH probe. Rhodamine dyes are used because of their superior lipophilicity and membrane permeability. The spirocyclic rhodamines are nonfluorescent at basic and neutral pH conditions. Upon changing the pH to acidic conditions, the spirocyclic forms transform into ring-opening forms with strong fluorescence emission. Rhodamine derivatives have been reported to specifically accumulate in lysosomes, and many rhodamine derivates have been developed for monitoring the lysosomal pH alterations of living cells. Rhodamine derivatives (e.g., Rhodamine 123) have also been reported as mitochondrial-targeting moieties. Coumarins are ideal candidates for developing cellular and molecular imaging tools because of their good cell permeability and minimal interference with living systems. The emission profile of coumarin dyes can be tuned from blue to near-infrared regions by simply modifying the substituents. Under acidic and basic conditions, the two fluorescent moieties exhibit different excitation and/or emission maxima. Azide–alkyne “click chemistry” was used to construct the linkage between the rhodamine and coumarin derivatives, leading to a bridge with three triazole rings.

Our rationale underpinning the design of RCPP was straightforward: equipping the pH-responsive fluorophores (rhodamine- and coumarin-based derivatives) with the triazole moiety as an anchor that drives the entire molecule into the desired organelles. Triazoles are basic aromatic heterocyclic compounds, and they have been used as a surrogate of neutral aromatic amino acid side chains to enhance target binding affinity and bioavailability. The triazole moiety in our RCPP probe functions as (1) a linker between these two fluorophores; (2) a structural and electronic component to fine-tune the photophysical property of the entire molecule; and (3) an improved aromatic heterocyclic core for in vitro/in vivo imaging in relation to its unique thermodynamic stability.

Mitochondria-targeted coumarin derivatives have been reported. Recently, we have identified several triazole-tethered-coumarin derivatives that could be used as mitochondria-targeting moieties. It is therefore reasonable to hypothesize that the triazole-tethered-coumarin moiety may initially enable RCPP to gather in the mitochondria. Because the destination of exogenous entities is lysosome, the triazole-rhodamine moiety may eventually relocate the intact molecule to the lysosome. Mitochondrial and lysosomal functions are intricately related and critical for maintaining cellular homeostasis. Conjugation of these two fluorescent labels in the molecular design of RCPP would minimize their permeability differences, thereby providing a single probe that can monitor these two different organellar pH fluctuations in living cells. It would therefore be advantageous to observe that the fluorescent probe moves between mitochondrion and lysosome for real-time monitoring of pH fluctuation within these two different subcellular locations.

Synthesis and Characterization of RCPP. As outlined in Scheme 1, intermediate 1 was readily prepared via condensation between 2,4-dihydroxy benzaldehyde and N-acetylglycine following a protocol by Wang et al. with minor modification. Intermediate 1 was then trapped with sodium azide to produce 3-azido-7-hydroxycoumarin 2, which was then subjected to a “click reaction” with the alkyne derivative of rhodamine 4 to generate the desired rhodamine–coumarin pH probe, i.e., RCPP. Next, RCPP emission spectra were examined by varying pH buffers to characterize its properties.
and to determine its pH sensitivity. Stock solutions of RCPP were first dissolved in trace amounts of DMSO and then diluted with buffer solutions of varying pH values. Photostimulation of RCPP solutions at acidic pH with the light of 360 nm generates two fluorescence peaks at 475 and 575 nm that correspond to coumarin and rhodamine, respectively (Figure S1). The peak at 575 nm decreased from a pH of 4 to a pH of 7, while the peak at 475 nm significantly increased under these conditions. At pH 7, the rhodamine fluorescence (575 nm) had disappeared. Further increase of pH 7 to 9 further enhanced the coumarin fluorescence (475 nm). This data suggests that RCPP can be used as a probe within a broad pH range.

Intracellular Localization of RCPP. Next, we examined intracellular alterations of pH in RCPP-loaded HeLa cells. These studies were performed in RCPP-loaded HeLa cells treated with the K’/H’ ionophore nigericin. Nigericin is used to equilibrate intracellular pH and extracellular pH. HeLa cells were then fluorescently imaged at various pHs. In the preliminary study, we found that a longer incubation period (>1 h) of cells with a relatively high concentration of nigericin (>50 μM) indeed significantly increased the extent of mitochondria colocalization with lysosomes. In the use of 50 μM nigericin, the percentage of colocalization between these two organelles was ~51 ± 5.4%. In this case, it is nearly impossible to clearly differentiate mitochondria and lysosomes. As a result, both mitochondria and lysosomes seemed to emit the same color (red or blue) fluorescence of RCPP depending on the pH value of the extracellular medium. This type of artifact is most likely due to the increased frequency of mitochondrial translocation to lysosomes caused using high concentrations of nigericin. Most cellular events depend on a tightly compartmentalized distribution of H’ ions across membrane-bound organelles. Organelles are surrounded by one or two membranes that separate the contents from the rest of the cytoplasm. It is important to ensure the measurement of pH in defined organelles in intact cells. To minimize any perturbations upon organelles, a much lower concentration of nigericin (1 μM) was adopted for all of the following assays.

As many biological samples are inherently fluorescent, it is important to ensure that the fluorescent signal is from the molecular probe and not the background. To do so often, an excess of RCPP probe is added to the system; however, this may lead to overloading of the system and to crosstalk between channels. To avoid these issues, sequential excitation and detection of fluorochrome techniques were utilized in our confocal imaging analysis. Sequentially scanning cells with individual lasers and detecting fluorescence in each channel to avoid exciting both fluorophores simultaneously is an effective method to control bleed-through in confocal microscopy, particularly when the choice of emission filters is limited.

In early pilot studies, we found that RCPP featured high sensitivity and a fast response over a broad pH range (Figure S2). RCPP is constructed with two dyes possessing different pH sensitivities and emission colors. Thus, it is not surprising to observe a fluorescent color change from red in the acidic region to blue as the pH increased. Next, the intracellular localization of the RCPP probe was carefully examined using distinct organelle secondary markers. An overlap in fluorescence does not necessarily indicate the colocalization of two probes in the same cellular structure due to the limitation of image resolution. The significant advantages in image resolution and the detection techniques of a laser scanning confocal microscope made the colocalization analysis much more reliable at the subcellular level than the conventional fluorescence microscope. In our present study, the colocalization of the two probes in confocal image analysis was evaluated visually and quantitatively. The high percentage of the colocalization of two fluorescent dyes throughout the cell provides strong evidence that the two may reside in the same organelles. At a pH of 4, double staining of RCPP with LysoTracker (a lysosome selective stain) indicated subcellular localization of RCPP in lysosomes (Figure 1A). To further quantify the degree of colocalization of RCPP and LysoTracker, a quantitative analysis of confocal images was performed. The Pearson’s correlation coefficient (POC) and the Mander’s overlap coefficient (MOC) were used to quantify the degree of colocalization between fluorophores (pH 4). The Pearson’s correlation coefficients for RCPP and

Figure 1. Intracellular distribution of RCPP compared to MitoTracker at a pH of 9.0 and LysoTracker at a pH of 4. After washing three times with the corresponding pH buffers, HeLa cells stained with RCPP (10 μM) were incubated with nigericin (1 μM) and were subsequently imaged in buffers with a pH of 4.0 and 9.0. Subsequently, cells were counterstained with LysoTracker (2 μM) (A) or MitoTracker (2 μM) (B), respectively, and then imaged for fluorescence on an inverted laser scanning fluorescent microscope (Olympus) using a 60x oil immersion objective lens. All pseudocolor images were captured using Olympus standard excitation and emission filter sets (λex/λem: 405/419–465, 488/500–550, and 592.5/608–646 nm).
LysoTracker were relatively high. Additionally, the overlap coefficients of the two fluorescence patterns were also high (Table S1). At a pH of 9, a double stain of RCPP with LysoTracker resulted in a distinct labeling pattern that was
These studies were followed by double staining with MitoTracker, which further indicated that the subcellular localization of RCPP at a pH of 9 is within the mitochondria (Figure 1B). POC and MOC were used to quantify the degree of colocalization between RCPP and MitoTracker (Table S1). The observation...
of repeated evidence for the colocalization of the RCPP probe and MitoTracker in mitochondria throughout the cell enhances our confidence that the two dyes reside in the same subcellular structure.

In response to extracellular pH alterations, organelles continuously adjust their numbers, size, and intracellular location.\(^{21,22,56-58}\) We examined lysosomal pH and morphological changes using RCPP and LysoTracker. At a pH of 6.4, lysosomes were more dispersed and appeared mainly as small puncta. The majority of lysosomes were located closer to the periphery of cells with weak red fluorescence in the presence of RCPP. By lowering pH values (6.4 → 4.0), the number of vesicular lysosomes with stronger red fluorescence of RCPP increased gradually and the mean volume of each lysosome increased (Figure 2). In addition, the total volume of lysosomes also increased significantly. We also noticed that lysosomes mobilize from the cell periphery to the perinuclear region and that the majority of cells displayed the strong red fluorescence of RCPP.

Similarly, we noticed that HeLa cells underwent morphological changes as a function of extracellular pH. At a pH of 7.6–8.4, mitochondria were equally dispersed throughout the cell with the weak blue fluorescence of RCPP residing within a filamentous network. An increase of pH (8.8–9.2) induced significantly more dispersed and irregular staining patterns with strong blue fluorescence for RCPP. Treatment of HeLa cells at a higher pH (9.6–10.0) resulted in mitochondrial localization to the perinuclear region with stronger blue fluorescence for RCPP. The mitochondria also adopted a

Figure 4. Proposed action mechanism of RCPP as a mitochondrial/lysosomal pH probe.
perinuclear cluster, and necklace-shaped rings remained near the nuclear periphery (Figure 3).

The coumarin moiety in our RCPP is a 7-hydroxycoumarin derivative. 7-Hydroxy-coumarin (also called umbelliferone) changes absorption and fluorescence spectra with pH. The absorption and fluorescence maxima for the coumarin chromophore are slightly shifted to longer wavelengths in RCPP because of the linker. The main reason for the pH response of the coumarin chromophore is the shift in the absorption spectrum with pH. Under basic conditions, the neutral form of RCPP is transformed to an anionic form with the oxygen-centered anion. Under the acidic condition, the spirolactam form of RCPP is converted to the ring-opened form (Figure 4). The absorption and fluorescence are greatly enhanced as a result of the ring-opening process.

Initially, the triazole-conjugated coumarin moiety may target RCPP to mitochondria. The slightly alkaline microenvironment of mitochondria promotes the conversion from the neutral form to the anionic form of RCPP. Accordingly, the triazole-conjugated coumarin moiety emits blue fluorescence for mitochondria labeling. Because of the ability of lysosomes to endocytose, it is reasonable to propose that RCPP might be trapped by lysosomes. In the acidic lysosome microenvironment, the spirolactam form of RCPP is converted to the ring-opened form. Thus, lysosomes can be labeled with the red emission of triazole-linked rhodamine moiety. As such, RCPP can be applied for simultaneously monitoring mitochondrial lysosomal pH values in association with different fluorescence patterns (Figure 4).

In summary, we have designed and synthesized a novel broad range pH probe (RCPP) by integrating an acidic pH-responsive rhodamine label with an alkaline pH-responsive coumarin moiety. Depending on the organellar acidity/alkalinity, RCPP may simultaneously move between mitochondrion and lysosome, two different subcellular locations. Thus, we concluded that RCPP possesses high dual-organelle-targeting selectivity. We further confirmed that RCPP could be used for simultaneous monitoring of acidity/alkalinity alterations in mitochondria (blue fluorescence) and lysosomes (red fluorescence) in living cells. RCPP works well in terms of organellar acidity/alkalinity monitoring at large deviations from physiological pH, both in terms of acidic (red fluorescence) and basic conditions (blue fluorescence). Nonetheless, limited studies have shown that, at subtle deviations from physiological conditions (pH ∼7.0–8.0), the pH monitoring capacity of RCPP is not as robust. Our current RCPP prototype will serve as a starting compound from which molecular alterations can be introduced that lead to a pH probe with the capacity to monitor more subtle variations of organellar pH from the nonphysiological region.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03087.

NMR spectra of the synthetic compounds, fluorescence spectroscopy, and cell imaging (PDF)

AUTHOR INFORMATION

Corresponding Authors

K. Michael Gibson — Department of Pharmacotherapy, College of Pharmacy and Pharmaceutical Sciences, Washington State University, Spokane, Washington 99202, United States; Email: mike.gibson@wsu.edu

Lanrong Bi — Department of Chemistry, Michigan Technological University, Houghton, Michigan 49931, United States; orcid.org/0000-0001-6624-8314; Email: lanrong@mtu.edu

Authors

Nazmiye B. Yapici — Department of Chemistry, Michigan Technological University, Houghton, Michigan 49931, United States

Xiang Gao — Department of Chemistry, Michigan Technological University, Houghton, Michigan 49931, United States

Xin Yan — Department of Chemistry, Michigan Technological University, Houghton, Michigan 49931, United States

Shanshan Hou — Department of Chemistry, Michigan Technological University, Houghton, Michigan 49931, United States

Steffen Jockusch — Department of Chemistry, Columbia University, New York, New York 10027, United States; orcid.org/0000-0002-4592-5280

Lillian Lesniak — Department of Chemical Engineering, Michigan Technological University, Houghton, Michigan 49931, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c03087

Notes

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