Mitochondria-targeted ratiometric fluorescent probe for real time monitoring of pH in living cells

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Pyridinium functioned 7-hydroxy coumarin was presented as the first mitochondria-targeted ratiometric fluorescent probe CP for real time monitoring pH in living cells. Compared with commercially available mitochondrial trackers, CP possesses high specificity to mitochondria in living cells as well as good biocompatibility. Meanwhile, CP displays excellent pH sensitivity and anti-interference capability. Confocal image experiments confirm that CP can monitor mitochondrial pH changes associated with the mitochondrial acidification, cellular apoptosis and stress response efficiently in real time.

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1. Introduction

As a vital energy supplying organelle [1], mitochondria play significant roles in metabolism of cells, organs, tissues, as well as the whole living body including cell signaling via reactive oxygen species production [2], regulation of Ca2+ homeostasis [3,4], and the triggering of cell death [5]. The microenvironment of mitochondria is closely related to its function, especially pH which can influence all biochemical processes directly. For example, under physiological conditions, mitochondria maintain an alkaline matrix (pH ~ 8), the proton-motive potential across the inner membrane drives ATP synthesis and ion and metabolite uptake into the matrix [6]. Meanwhile, the proton motive force further acts to regulate Ca2+ homeostasis [7,8], which in turn modulates dehydrogenase activity associated with the tricarboxylic acid (TCA) cycle [9], as well as adenine nucleotide translocase [10] and ATP synthase [11].

It is no doubt that small changes of mitochondrial pH will significantly influence its biologic functions. On the other side, production of excess reactive oxygen species (ROS) lead to mitochondrial autophagy and apoptosis which can further induce mitochondrial acidification [12]. Moreover, abnormal levels of mitophagy are closely related to various pathological conditions, including cardiovascular diseases [13], neurodegenerative diseases [14], and Reye’s syndrome [15]. Therefore, real-time detailed monitoring of mitochondrial pH changes is urgently necessary, which have significant effect on mitochondrial biology and associated diseases.

As an excellent detection technique, fluorescent probes have attracted increasing attention due to their high selectivity, excellent sensitivity and real-time and high spatial resolution imaging in living cells or organisms. As far as we know, there are only a few examples reported for the real-time monitoring of mitochondrial pH changes in intact cells including green fluorescent proteins (GFPs) modified with mitochondrial targeting peptides [6,16,17]. Compared with these protein probes, small fluorescent probes are expected to be more readily applicable for use in native cells with less interference from the organism. Early this year, Tang reported the first single wavelength near-infrared-emitting fluorescent probe for monitoring mitochondrial pH [18]. More recently, Kim reported a mitochondria-immobilized pH-sensitive off-on
fluorescent probe [19]. Compared to these intensity-based fluorescence sensors, ratiometric fluorescent probes can efficiently exclude interference with signal output by concentration, instrumental efficiency, and environmental conditions through ratiometric self-calibration of the two emission bands to allow an accurate and quantitative measurement [20]. Disappointedly, no ratiometric fluorscencs probe for monitoring mitochondrial pH have been reported yet. Herein, we would like to present the first ratiometric fluorescent probe for real-time monitoring mitochondrial pH.

Previously, we described a water-soluble near-infrared probe for ratiometric sensing of SO2 based on the 7-hydroxy coumarin and TCF [21]. The phenolic hydroyl group is a pH sensitive group: in acidic or neutral conditions, the hydroyl group is a weak electron-donating group; however the deprotonated O− is a much stronger electron-donating group under alkaline conditions. Protonation or deprotonation of the phenolic hydroyl group can result in the fluorescent emission spectrum blue-shift or red-shift, respectively [22,23]. As a double membrane organelle, the membrane electrical deprotonation of the phenolic hydroxyl group can result in the electron-donating group under alkaline conditions. Protonation or deprotonation of the phenolic hydroyl group can result in the fluorescent emission spectrum blue-shift or red-shift, respectively [22,23].

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2. Experimental section

2.1. Materials and general instruments

Mito Tracker Deep Red and Lysotracker Deep Red were purchased from Invitrogen. Mitochondrial membrane potential assay kit (TMBE, ab113852) was purchased from abcam®. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. All the solvents were dried according to the standard methods prior to use. All the solvents were either HPLC or spectroscopic grade in the optical spectroscopic studies. TLC analyses were performed on silica gel GF 254. Column chromatographic purifications were carried out on silica gel (HG/T2354-92). NMR spectra were measured on a Bruker Avance III 500 spectrometer. Fluorescence excitation and emission spectra were obtained using FluoroMax-4 Spectrofluorimeter (HORIBA Jobin Yvon). UV-Vis absorption spectra were recorded on a Hitachi PharmaSpec UV-1900 UV-Visible spectrophotometer. All pH measurements were performed with a pH-sensitive digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode.

2.2. Preparation of the test solutions

A stock solution of CP (1.0 mM) was prepared in DMSO. All the stock solutions of cations, anions, H2O2 and reactive sulfur were prepared from the corresponding phosphoric acid, and 40 mM acetic acid were used for tuning pH values. All samples for fluorescence experiments were performed in different pH 3-10 buffer solution for 30 s before measurement.

2.3. Quantum yields

Quantum yields were determined using fluorescein as a standard according to published method [37]. The quantum yield was calculated according to the equation: \( \Phi_{\text{Sample}} = \frac{\Phi_{\text{Standard}} \times (\text{Sample \\text{Abs}_{\text{em}}}/\text{Standard \\text{Abs}_{\text{em}}})}{0.85} \), where \( \Phi \) is the quantum yield, \( \Phi_{\text{Standard}} = 0.85 \) in 0.1 M NaOH; \( \text{Sample \\text{Abs}_{\text{em}}} \) and \( \text{Standard \\text{Abs}_{\text{em}}} \) are the integrated fluorescence intensities of the sample and the standard, \( \text{Sample \\text{Abs}_{\text{em}}} \) and \( \text{Standard \\text{Abs}_{\text{em}}} \) are the optical densities, at the excitation wavelength, of the sample and the standard, respectively.

2.4. Synthesis of CP

95 mg (0.5 mmol) of 7-hydroxy coumarin aldehyde, 171.5 mg 1,4-dimethylpyridin-1-ium iodide (0.5 mmol, 1.0 equiv) and 50 μL piperidine were dissolved in 20 mL EtOH, the mixture was stirred under reflux for 12 h, a deep red solid was obtained through filtration and recrystallization from EtOH. The crude product was purified by chromatography on a silica gel column using DCM: MeOH = 2:1 to MeOH as the mobile phase, affording CP as a red powder 130 mg (63.9% yield), 1H NMR (400 MHz, DMSO-d6), δ 8.53 (d, 2H, J = 6.8 Hz), 7.89 (d, 2H, J = 7.2 Hz), 7.33 (d, 1H, J = 15.6 Hz), 7.12 (d, 1H, J = 8.8 Hz), 6.10 (dd, 1H, J = 2.0 Hz, 8.8 Hz), 5.79 (dd, 1H, J = 0.8 Hz, J = 2.0 Hz), 4.11 (S, 1H); 13C NMR (100 MHz, DMSO-d6), δ 180.8, 160.4, 158.8, 153.8, 144.0, 143.6, 139.5, 130.7, 122.3, 121.1, 115.9, 106.3, 103.9, 103.4, 45.8. HRMS (ESI): [m/z] [M]+ calcd for C17H13NO7S: 280.0968; found 280.0966.

2.5. CCK-8 assay for the cell cytotoxicity

Toxicity toward Hela cells was determined by cell counting kit-8 (CCK-8). About 7000 cells per well were seeded in 96-well plates and cultured overnight for 70-80% cell confluence. The medium was replaced with 50 μL of fresh medium, to which 50 μL CP at various concentration was added to achieve final volume of 100 μL. Twenty-four hours later, 10 μL CCK-8 mixed in 90 μL PBS was added to each well for additional 1 h incubation. The absorbance was measured in an ELISA plate reader (model 550, BioRad) at a wavelength of 450 nm. The metabolic activity of the polypeptides treated cells was expressed as relative to untreated cell controls taken as 100% metabolic activity.

2.6. Cell culture and fluorescence imaging

Hela cells were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS (Fetal Bovine Serum) and 1% Antibiotic-Antimycotic at 37 °C in a 5% CO2/95% air incubator. For fluorescence imaging, cells (4 × 103/well) were passed on confocal dishes and incubated for 24 h. Fluorescence imaging was performed with a LEICA TCS-SP5 Laser Scanning Confocal Microscope with a 40 × oil-immersion objective lens.

2.6.1. Colocalization experiments

Hela cells were incubated with CP (5 μM) in culture medium for 30 min at 37 °C, and then cells were washed with PBS three times. 1.0 μM Mito tracker Deep Red or LysoTracker Deep Red was added and co-incubate for another 30 min and cell imaging was then carried out after washing cells with physiological saline three times. Emission was collected at 460 – 560 nm (excited at 405 nm) for green channel and 580 – 650 nm (excited at 488 nm) for red channel. Mit tracker Deep Red or Lyso tracker Deep Red was collected at 660 – 740 nm (excited at 633 nm) for near-infrared region which was marked with blue color.

2.6.2. Mitochondria pH monitoring and CCCP (m-chlorophenyl hydrazone) treatment

Hela cells were pretreated with media containing CP (5.0 μM) for 30 min. The cells were washed three times with PBS. And then the media were replaced with PBS containing different drugs and incubated for another 30 min or 1 h at 37 °C. Fluorescent confocal images were then recorded using an excitation wavelength of 405 nm (λem = 460 – 560 nm) and 488 nm (λem = 580 – 650 nm).

2.6.3. Intracellular pH calibration

Hela cells were pretreated with media containing CP (5.0 μM) for 30 min. The cells were washed three times with PBS and then the cells were incubated with high K+ buffer (30 mM NaCl, 120 mM KCl, 1 mM CaCl2, 0.5 mM MgSO4, 1 mM Na2HPO4, 5 mM glucose, 20 mM HEPES, and 20 mM NaOAc) at various pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0) in the presence of 5.0 μM of nigericin for 20 min at 37 °C. The fluorescence images were captured, and the pH calibration curve was constructed with a confocal microscope.

3. Results and discussion

3.1. Synthesis and characterization of the fluorescent probe CP

Through the condensation of 4-methyl pyridinium with 7-hydroxy coumarin-3-carbaldehyde in EtOH with the catalyst of piperidine (Scheme 1) for 12 h, CP was obtained via filtration in 63.9% yield. The structure was confirmed by 1H NMR, 13C NMR and HRMS (see supporting information).
3.2. Optical Properties of CP

The UV-Vis absorption and fluorescent emission spectroscopy of CP (10 μM) was investigated in B–R buffer solution (40 mM acetic acid, phosphoric acid, and boric acid) at various pH values (Fig. 1). The probe displayed highly sensitive responses to the pH changes. As depicted in Fig. 1a, when the pH decreased from 10.0 to 4.0, the maximum absorption band at 467 nm of CP in pH 10.0 media was blue-shifted to 400 nm in pH 4.0 (Fig. 1a). And in pH 7.0, there were two emission spectroscopies at 528 nm (excitation at 400 nm) and 606 nm (excitation at 467 nm) which were corresponding to the products of protonation and deprotonation respectively. When the pH increased from 4.0 to 10.0, the peak at 606 nm raised dramatically with a concomitant decrease in the peak at 528 nm (Fig. 1c and d); this provided the basis for achieving a ratiometric detection (Fig. 1b). A plot of the fluorescent ratio at F_{528}/F_{606} vs pH revealed a linear relationship over the pH range of 5.0–7.0. The pKa value of CP is 5.88 [38]. The quantum yields in acid or basic condition are 0.0584 at pH 4.0 and 0.0159 at pH 9.0 respectively.

**Scheme 1.** The synthesis of mitochondria-targeted pH-sensitive fluorescent probe CP.

**Fig. 1.** Ultraviolet (a) and fluorescence (c, d) titration of CP (10 μM) in 40 mM B–R buffer solution at various pH from 4.0 to 10.0 (for c: λ_{ex} = 400 nm, slit: 5 nm/5 nm; for d: λ_{ex} = 467 nm, slit: 5 nm/5 nm). (b) Plot of the fluorescent ratio at F_{528}/F_{606} vs pH.
As we all know that the intracellular environment is complicated with many different biomolecules and ions. Meanwhile, as a good pH fluorescent probe, excellent anti-interference capacity is a key factor. To confirm that CP could measure the pH value efficiently among the complicated endocellular environment, we measured the fluorescence spectra of CP in the presence of essential cations, anions, and oxidative-stress-associated redox chemicals (Cys, Hcy, GSH and H2O2) at pH 7.0 for the existing of both species at the same time (protonation and deprotonation). As can be seen from Fig. 2a (Fig. S1), almost no obvious spectroscopic and ratio changes are observed whether in the presence of additional anions, cations as well as the oxidative-redox species. Some reactive sulfur species like S²⁻, SO₃⁻, Cys, Hcy and GSH usually used as the Michael addition species to the α, β-unsaturated bond revealed little influence even at the concentration of 1 mM of Cys and GSH, which indicates the high selectivity of CP for pH detection. Moreover, CP shows excellent reversibility between pH 4.0 and 8.0 in 20 mM B–R buffer solution due to the protonation/deprotonation of the hydroxyl group (Fig. 2b).

### 3.3. Cytotoxicity measurements

Encouraged by the aforementioned results, we further explored the capacity of CP for the fluorescent imaging in living cells. First, a standard CCK-8 assay for Hela cell was investigated at different concentration of CP (Fig. 3). The results showed that even when incubated with 5 μM CP for 24 h, about 90% of Hela cells survived, which indicated the low cytotoxicity of CP compared with other reported mitochondrial targeting pH fluorescent probe [18,19].

### 3.4. Fluorescence imaging of CP in the living cells

In order to confirm the presumed mitochondrial specificity of CP, co-localization experiments were performed in Hela cells using a commercially available mitochondrial specific near-infrared fluorescent dye, Mito Tracker Deep Red (MTDR, 1.0 μM). As expected, the fluorescence image of CP (5 μM) both in the green and red light district overlaps very well with that obtained using MTDR (blue) (Pearson’s correlation coefficient: 0.93). Meanwhile, co-localization experiments were also performed in 293T cells to further confirm mitochondrial specificity of CP (Fig. S2). The changes in the intensity profile of linear regions of interest (ROIs) (CP and MTDR costaining) tended toward synchronization (Fig. 4).

However, a poor overlap was observed between the fluorescence of CP (5 μM) and a lysosome-specific near-infrared dye (LTDR, 1.0 μM) (Pearson’s correlation coefficient: 0.78), which suggesting that CP could specifically target to mitochondrial in living cells (Fig. 5). Meanwhile, by using CP, we can observe the microminiature clavate shape of mitochondrial (Fig. 6) [39].

Due to the wonderful biocompatibility and mitochondrial specificity, in the following experiments CP was utilized to monitor mitochondrial pH changes in real time. As mentioned above, mitochondria play important roles in maintaining genomic integrity because they continuously oxidize substrates and maintain a proton gradient across the lipid bilayer with a very large mitochondrial membrane potential (ψm) of around −180 mV. After incubating with 5.0 μM CP for 30 min, Hela cells were stimulated by different amounts (0, 10, 20, 50 μM) of carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a typical uncoupler of oxidative phosphorylation that abolishes the mitochondrial membrane proton gradient to change the membrane potential (ψm) of mitochondrial during the staining procedure [40]. Fig. 7 revealed the fluorescence intensity decreased significantly in both the green and red channels with the addition of CCCP compared with cells only incubated with CP. Furthermore, the fluorescence intensity decreased gradually...
when the amount of CCCP was increased from 10 μM to 50 μM. These results demonstrated that when exposed to CCCP, the mitochondrial membrane potential was out of balance (which could be confirmed by tetramethyl rhodamine ethyl ester as indicator, Fig. S3). Once the cells selectively removed the damaged mitochondria, the mitochondrial targeting agent (CP) would be obliterated out of cells. At the same time, the fluorescent intensity from the green channel is superior to that in red channel which is opposite without CCCP. We can observe that the protonophore CCCP rapidly induced an obvious acidification of mitochondria which may be attributed to mitochondrial apoptosis when treated with CCCP.

Fig. 4. Colocalization imaging of Hela cells stained with CP and Mito Tracker Deep Red (MTDR). The cells were incubated with CP (5.0 μM) for 30 min at 37 °C, and the medium was replaced with fresh medium containing MTDR (1.0 μM) and incubated for another 30 min. (a) Confocal image from CP on Green channel (λex = 405 nm, λem = 460–560 nm); (b) Confocal image from CP on red channel (λex = 488 nm, λem = 580–650 nm); (c) Confocal image from MTDR on near-infrared channel (λex = 633 nm, λem = 660–740 nm); (d) Bright-field image. (e) Merged image of (a) and (c). (f) Merged image of (b) and (c). (g) Merged image of (a), (b), and (d). (h) Intensity profile of ROIs across Hela cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Colocalization imaging of Hela cells stained with CP and Lyso Tracker Deep Red (LTDR). The cells were incubated with CP (5.0 μM) for 30 min at 37 °C, and the medium was replaced with fresh medium containing LTDR (1.0 μM) and incubated for another 30 min. (a) Confocal image from CP on Green channel (λex = 405 nm, λem = 460–560 nm); (b) Confocal image from CP on red channel (λex = 488 nm, λem = 580–650 nm); (c) Confocal image from LTDR on near-infrared channel (λex = 633 nm, λem = 660–740 nm); (d) Bright-field image. (e) Merged image of (a) and (c). (f) Merged image of (b) and (c). (g) Merged image of (a), (b), and (d). (h) Intensity profile of ROIs across Hela cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
It was reported that lactate plus pyruvate can cause mitochondria acidification by diffusion of protonated acid or by co-transport of pyruvate $\mathrm{^\circ}/\mathrm{H}^+$ through the inner mitochondrial membrane [6]. So CP was used to capture the mitochondrial pH changes with additional of 10 mM lactate plus 1 mM pyruvate in Hela cells (Fig. 8). Compared with the cells treated with 5.0 $\mu\mathrm{M}$ CP merely, the fluorescence intensity of the stimulated cells decreased in the red channel following a concomitant increase in green channel obviously. The quantitative fluorescence intensity ratio of green and red channels displayed the pH decrease (Fig. 8).

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**Fig. 6.** Confocal image of CP (5.0 $\mu\mathrm{M}$) in mitochondria.

**Fig. 7.** Confocal image of CP (5.0 $\mu\mathrm{M}$) incubated with 0, 10, 20 and 50 $\mu\mathrm{M}$ CCCP (Carbonyl cyanide m-chlorophenylhydrazone) in Hela cells from green channel (a, e, i, m, $\lambda_{\text{ex}} = 405$ nm, $\lambda_{\text{em}} = 460–560$ nm), red channel (b, f, j, n, $\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 580–650$ nm), bright-field (c, g, k, o) and merged (d, h, l, p). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 8. Confocal image of CP (5.0 µM) incubated with 10 mM lactate plus 1 mM pyruvate in Hela cells for 30 min from green channel (a, e, λex = 405 nm, λem = 460–560 nm), red channel (b, f, λex = 488 nm, λem = 580–650 nm), bright-field (c, g) and merged (d, h). (i) Quantification of fluorescence intensity ratio of green and red channel from CP and CP + P + L (pyruvate plus lactate). [For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.]

Fig. 9. Confocal image of CP (5.0 µM) incubated with 0.1 mM H2O2 or 1 mM NAC (N-acetylcysteine) in Hela cells for 1 h from green channel (a, e, i, λex = 405 nm, λem = 460–560 nm), red channel (b, f, j, λex = 488 nm, λem = 580–650 nm), bright-field (c, g, k) and merged (d, h, l). (m) Quantification of fluorescence intensity ratio of green and red channel CP, CP + H2O2 and CP + NAC. [For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.]

Fig. 10. Confocal image of CP (5.0 µM) incubated with 30 µM chloroquine in Hela cells for 1 h from green channel (a, e, λex = 405 nm, λem = 460–560 nm), red channel (b, f, λex = 488 nm, λem = 580–650 nm), bright-field (c, g) and merged (d, h). (i) Quantification of fluorescence intensity ratio of green and red channel from CP and CP + CQ (chloroquine). [For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.]
Mitochondria regulate the cellular redox state and a breakdown in mitochondria potential leads to the production of reactive oxygen species (ROS), which act as signaling molecules to initiate apoptosis \[12\]. Therefore, different redox substances were applied to explore the relationship between mitochondrial pH and oxidative stress. First, Hela cells were exposed in 100 \(\mu\)M \(\text{H}_2\text{O}_2\) for 1 h with the staining of CP. As shown in Fig. 9, the introduction of \(\text{H}_2\text{O}_2\) led to a gradual mitochondrial acidification. The average ratio...

**Fig. 11.** Confocal image of CP (5.0 \(\mu\)M) in Hela cells clamped at pH 4.0 (1–4), 4.5 (5–8), 5.0 (9–12), 5.5 (13–16), 6.0 (17–20), 6.5 (21–24), 7.0 (25–28). For green channel (1, 5, 9, 13, 17, 21, 25, \(\lambda_{\text{ex}} = 405\) nm, \(\lambda_{\text{em}} = 460–560\) nm), red channel (2, 6, 10, 14, 18, 22, 26, \(\lambda_{\text{ex}} = 488\) nm, \(\lambda_{\text{em}} = 580–650\) nm), bright-field (3, 7, 11, 15, 19, 23, 27) and merged (4, 8, 12, 16, 20, 24, 28). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
(\(F_{\text{green}}/F_{\text{red}}\)) increased from 0.70 to 3.00 which may be ascribed to the generation of acidic substances from the hydrolysis of intracellular ATP [41]. Similar redox substances NAC (GSH precursor, 1 mM) were incubated with HeLa cells to investigate the influence of GSH on the mitochondrial pH. However, no obvious change in either the green or red channels was observed, implying that GSH over the normal concentration may not cause a remarkable change in the concentration of acidic substances within the mitochondria. The quantitative fluorescence intensity ratio between the green and red channels displayed very a weak pH increase (the average ratio decreased from 0.70 to 0.61).

As is known to all, in the process of the whole life, all the organelles, tissues, and organs are interacting with each other to maintain the operation of whole life activities. Damages of any one organelle may affect the operation of other organelles. So we would like to try to explore if harm to the lysosome could affect normal mitochondria physiological activities. As an antimalarial agent, chloroquine is a typical lysosomal toxin [42]. Chloroquine can lead to the alkalization of the lysosome. Hence, Hela cells were exposed in 30 \(\mu M\) chloroquine for 1 h after the staining with CP (Fig. 10). Compared with cells only staining with CP, the fluorescence intensity in the green channel is apparently stronger then the red channel, which suggests that chloroquine may result in the acidification of mitochondria (Fig. 10i). This could be ascribed to the respiration inhibition and the corresponding uncoupling of oxidative phosphorylation of chloroquine. Studies have shown that people who always take chloroquine usually have the “oxygen lack” phenomenon [43]. Furthermore, mitochondrial acidification is also seen during the mitophagic elimination (mitophagy) of malfunctioning mitochondria through lysosomal fusion [44,45].

Finally, the fluorescent response of CP to pH changes in living HeLa cells was tested using confocal microscopy imaging (Fig. 11). The intracellular pH calibration curve was obtained using high K\(^+\) buffer and ionophore nigericin (5.0 \(\mu M\)) in a variety of pH values [46,47]. The fluorescence emission intensity in the green channel decreased with an increase in the cellular pH from 4.0 to 7.0 generally, while the fluorescence emission intensity in the red channel gradually increased which matched well with the fluorescent titration in different pH solutions (Fig. 11). The changes in the ratio of green to red intensity exhibited a pH-dependent signal linearly over the pH range 4.5–6.5 (\(R = 0.9877\), Fig. 12). Although this linear pH range is out of the normal mitochondrial pH value, we still believe that CP can be used for monitoring pH qualitatively and quantitatively in damaged mitochondria or in pathogenic state in living cells.

### 4. Conclusion

In summary, we have presented a novel water-soluble fluorescent probe (CP) that combines the 7-hydroxy coumarin and pyridinium platforms. This probe exhibits excellent pH sensitivity and extraordinary anti-interference capability with biologically-relevant cations, anions, reactive sulfur species and oxidative-stress-associated redox chemicals. Meanwhile, with good biocompatibility, CP was further used for ratiometric fluorescence imaging in living cells with excellent mitochondrial targeting ability. We revealed that CP permits the real time monitoring of pH changes associated with the mitochondrial acidification, cellular apoptosis or stress response. It is the first ratiometric fluorescence probe for monitoring mitochondrial pH changes in living cells. And for the first time, we investigated the influence of chloroquine with lysosomal toxicity to the physical activity of mitochondrial. This may supply important evidence for the interaction between mitochondria and lysosome in vital movement. Moreover, the proposed method may provide a new strategy for the quantitative mitochondrial pH via a ratiometric fluorescence probe in future.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.02.113.

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