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# A cell-penetrating ratiometric probe for simultaneous measurement of lysosomal and cytosolic pH change

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ABSTRACT

A new ratiometric fluorescent probe based on cell-penetrating peptides (CPPs) was constructed for whole-cell pH mapping and simultaneous measurement of pH changes in the cytoplasm and lysosomes. The arginine-rich CPP, $R_{12}K$ worked as linker, carrier and part of the fluorophore. Benefiting from $R_{12}K$, the fluorescent probe is completely water soluble, membrane permeable and well biocompatible. It shows high selectivity, sensitivity and reversibility to pH fluctuations. The ratio of fluorescence intensities $F_{519}/F_{582}$ increased from 0.2 to 9.2 over the pH range from 3.3 to 8.1. Intracellular pH mapping was successfully realized owing to the wide distribution of the probe in live cells (even in nucleus). Moreover, cytosolic and lysosomal pH change caused by the stimuli can be simultaneously detected. Compared to other ratiometric pH probes, RhB-$R_{12}$K-FITC can provide more precise information about $H^+$ redistribution between different cellular compartments.

## 1. Introduction

Cells are highly compartmentalized and organized. Different cellular compartments with varied pH values provide distinct conditions for optimal operation [1]. The lumens of lysosomes and endosomes are faintly acidic [2,3], whereas nucleus and cytoplasm are near neutral [4,5]. The pH changes of lysosomes and cytoplasm are closely related in basic cellular activities, such as metabolism [1], oxidative stress [6] and apoptosis [7,8]. Abnormal pH values can lead to cellular dysfunction and indicate the risk of cancer [9–11], neurodegenerative disorders [12,13] and cardiovascular disease [14]. Monitoring intracellular pH homeostasis and how pH is regulated can improve our understanding of physiological and pathological processes.

Fluorescent imaging has occupied a decisive position in live cell imaging attributed to their high selectivity, excellent sensitivity and spatiotemporal resolution [15–18]. Ratiometric fluorescent probes give more accurate analysis on intracellular pH, because of the corrections of some systematic errors resulted from dye leakage, photobleaching and optical path length. Although various small-molecule probes and nanoprobes were developed for ratiometric pH sensing [19–27], most of them focus on one specific organelle. For in-depth research on $H^+$ redistribution in physiological process and cellular behavior, simultaneous pH analysis of different cellular compartments is urgently required. Using a combination of multiple organelle-specific pH sensors is much more complicated [28] and researchers are devoted to developing effective pH indicators for intracellular pH mapping [19,21,29]. As far as we know, few pH probes allow simultaneous detection of cytosolic and lysosomal pH change.

Cell penetrating peptides (CPPs) have been promising carriers to facilitate cellular internalization [30,31]. Our group previously reported a spirolactam derivative RhB-$R_{12}$K by conjugating the fluorescent dye rhodamine B to CPP [32]. Here, we fully took advantage of the remaining active groups in the CPP and constructed a ratiometric fluorescent pH probe RhB-$R_{12}$K-FITC. The new probe, which shows high selectivity and sensitivity to $H^+$, possesses excellent membrane permeability, water-solubility and low toxicity. It can even be phagocytized by nucleus due to the typical feature of arginine-rich CPPs [33]. Its remarkable properties and wide distribution in live cells enable relatively successful intracellular pH mapping. The pH value of specific compartment can be measured individually. Furthermore, the pH fluctuations and $H^+$ redistribution between cytoplasm and lysosomes caused by oxidative stress are verified.

## 2. Experimental

### 2.1. Reagents and apparatus

The CPP-based probe RhB-$R_{12}$K-FITC was synthesized, high efficiency liquid chromatography (HPLC) purified and characterized with ESI-MS by Shanghai Top-peptide Co., Ltd. (Shanghai, China). The stock solution of the probe was prepared by dissolving the powder in ultrapure water to 1 mM. Glutathione (GSH), N-Ethylmaleimide (NEM), N-

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acetylcysteine (NAC), chloroquine and all metal chlorides of analytical grade were purchased from Sigma Aldrich and used without further purification·$H_2O_2$ was obtained from Beijing Chemical Works (Beijing, China). Nigericin was purchased from J & K Chemical Technology (Beijing, China). Dulbecco's modified eagle media (DMEM), Dulbecco's modified eagle media without phenol red, fetal bovine serum (FBS), penicillin and streptomycin (100 U/mL), Trypsin EDTA and phosphate buffered saline (PBS) solution were purchased from GIBICO (Invitrogen, USA). Organelle specific dyes LysoTracker Blue DND-22 and MitoTracker Deep Red FM were purchased from Molecular Probes (Invitrogen, USA). 3-(4, 5-dimethyl-2-thiazolyl) − 2, 5-diphenyl-2-H-tetrazolium bromide (MTT) and Albumin Bovine V (BSA) were purchased from Biodee Biotechnology (Beijing, China). Ultrapure water (over 18 kΩ) from Milli-Q water purification system (Millipore) was used throughout the experiment. HeLa cells were obtained from Peking Union Medical College Hospital (Beijing, China). The absorption spectra were recorded with a U-3900 spectrophotometer (Hitachi, Japan). The Fluorescence spectra of RhB-$R_{12}$K-FITC at varied pH values and the interference of different species to the ratio of the probe were recorded with a F-7000 fluorescence spectrometer (Hitachi, Japan). The quantum yields were measured by a FLS920 steady state and transient state fluorescence spectrometer (Edinburgh Instruments, UK). The absorbance of MTT assay was measured by using a microplate reader M3 (Molecular Devices). Fluorescence imaging experiments were performed on a FV1000 confocal laser scanning microscope (Olympus, Japan) with a 60× objective lens. HeLa cells were incubated in a MCO-5AC $CO_2$ incubator (Panasonic, Japan). All buffer solutions were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). B-R buffer solutions at different pH values were achieved by adding NaOH or HCl to the mixture of 40 mM acetic acid, phosphoric acid and boric acid. High $K^+$ buffer solutions were made up of 120 mM KCl, 30 mM NaCl, 20 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $NaH_2PO_4$ and 0.5 mM $MgSO_4$.

### 2.2. Fluorescence spectral properties of RhB-$R_{12}$K-FITC

RhB-$R_{12}$K-FITC (2 μM) in B-R buffer solutions at varied pH values were used for pH calibration curve. Briefly, 2 μL RhB-$R_{12}$K (1 mM) and 998 μL B-R buffer solutions at specific pH value were mixed and the fluorescence spectra were recorded by a fluorescence spectrometer. The excitation wavelength was 488 nm.

The pH value of RhB-$R_{12}$K-FITC (4 μM) solution between 5 and 8 was adjusted back and forth by 5 M NaOH or HCl solution.

The interference of redox species (0.1 mM $H_2O_2$, 1 mM GSH, 1 mM NAC and 1 mM NEM), BSA (10 μM), $Na^+$ (1 mM), $K^+$ (1 mM) and some other metal ions ($Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Cu^{2+}$, $Fe^{3+}$, $Fe^{2+}$, $Al^{3+}$, $Zn^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Ba^{2+}$) (100 μM) to the relative ratio of RhB-$R_{12}$K-FITC (2 μM) at pH 4.9 and pH 7.4 was investigated, respectively. Briefly, 2 μL RhB-$R_{12}$K (1 mM) was added to 998 μL buffer solutions containing different metal ions. The fluorescence spectra were recorded by using a fluorescence spectrometer. The excitation wavelength was 488 nm.

### 2.3. Cell culture

HeLa cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL 1% penicillin and streptomycin (v/v) at 37 °C in a 5% $CO_2$ incubator. Cells were seeded in 15 mm confocal laser culture dishes and cultured in the medium for 24 h. The original medium was removed and cells were washed twice with PBS (pH = 7.4) before use.

### 2.4. Colocalization analysis

HeLa cells were incubated with RhB-$R_{12}$K-FITC (2 μM) in DMEM without phenol red at 37 °C for 50 min. The original medium was removed and the cells were washed twice with PBS. Then, HeLa cells were incubated with DMEM containing LysoTracker Blue DND-22 (50 nM) for another 30 min. Fluorescence imaging experiments were performed on a FV1000 confocal laser scanning microscope. The excitation wavelength of LysoTracker Blue DND-22 was 405 nm and the fluorescence signal was collected from 425 nm to 465 nm. The excitation wavelength of RhB-$R_{12}$K-FITC was 488 nm. The fluorescence signal of FITC part was collected from 505 nm to 540 nm and RhB part was collected from 575 nm to 655 nm.

HeLa cells were incubated with RhB-$R_{12}$K-FITC (2 μM) in DMEM without phenol red at 37 °C for 50 min. The original medium was removed and the cells were washed twice with PBS. Then, HeLa cells were incubated with DMEM containing MitoTracker Deep Red (100 nM) for another 30 min. Fluorescence imaging experiments were performed on a FV1000 confocal laser scanning microscope. The excitation wavelength of RhB-$R_{12}$K-FITC was 488 nm. The fluorescence signal of FITC part was collected from 505 nm to 540 nm and RhB part was collected from 580 nm to 615 nm. The excitation wavelength of MitoTracker Deep Red FM was 635 nm and the fluorescence signal was collected from 655 nm to 755 nm.

### 2.5. Intracellular pH calibration

HeLa cells were incubated with RhB-$R_{12}$K-FITC (2 μM) in DMEM without phenol red at 37 °C. After 50 min, the original medium was removed and the cells were washed with PBS three times. Then, the cells were incubated with high $K^+$ buffer solutions in the presence of nigericin (10 μM) at varied pH values in the incubator for 15 min. The fluorescence images were collected and analyzed with Olympus software (FV10-ASW). The fluorescence signal of FITC part was collected from 505 nm to 540 nm and RhB part was collected from 580 nm to 615 nm. The pH calibration curve was constructed according to the ratios of the selected regions of interest (ROIs), which were calculated pixel-by-pixel. All data were expressed as mean ± standard deviation.

### 2.6. Drug stimulation

HeLa cells were incubated with RhB-$R_{12}$K-FITC (2 μM) in DMEM without phenol red at 37 °C for 50 min. The medium was removed and the cells were treated with NAC (1 mM) for another 1 h. Fluorescence imaging experiments were performed on FV1000 confocal laser scanning microscope. The fluorescence signal of FITC part was collected from 505 nm to 540 nm and RhB part was collected from 580 nm to 615 nm. The pH values of lysosomes in selected ROIs were determined by the pH calculated curve. All data were expressed as mean ± standard deviation. HeLa cells treated with NEM (1 mM), chloroquine (200 μM) and $H_2O_2$ (100 μM) followed the same procedure.

### 2.7. MTT assay

HeLa cells were seeded in 96-well microtiter plates at a density of 8000 cells/well and cultured at 37 °C in a 5% $CO_2$ incubator for 24 h. The medium was removed and replaced with DMEM added the CPP-based probe RhB-$R_{12}$K-FITC (2 μM). The cells were incubated with RhB-$R_{12}$K-FITC for 1 h, 3 h, 6 h and 24 h, respectively. Then, 100 μL of the MTT solution (0.5 mg/mL) was added to each well. After 4 h, the MTT solution was abandoned and 100 μL of DMSO was added to each well to dissolve the formed formazan. The plates were shaken for 10 min and the absorbance at 490 nm was measured by a microplate reader M3.

## 3. Results and discussion

### 3.1. Synthesis and characterization of RhB-$R_{12}$K-FITC

The synthetic route of RhB-$R_{12}$K-FITC was outlined in Scheme 1. Rhodamine B (RhB) and fluorescein isothiocyanate (FITC) was conjugated to $R_{12}$K in DMF with the catalysis of O-benzotriazole-N, N, N′, N′-tetramethyluronium hexafluorophosphate (HBTU) and N-