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Single-wavelength Excited Ratiometric Fluorescence pH Probe to Image Intracellular Trafficking of Tobacco Mosaic Virus

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Abstract As a typical plant virus which has biocompatibility and high transfection efficiency, tobacco mosaic virus (TMV) has shown broad application potential in drug or gene delivery field. Elucidating its intracellular trafficking is of great importance in investigation of its cytotoxicity, targeting site, and delivery efficiency, and is advantageous to designing new TMV-based drug delivery systems with different targets. By taking advantage of the regulated pH value of different organelles in a mammalian cell, we exploit a pH detection strategy to investigate the intracellular trafficking pathway of TMV. Here, we report a single-wavelength excited ratiometric fluorescent pH probe. This probe is constructed by simultaneously coupling pH-sensitive fluorescein isothiocyanate (FITC) and pH-insensitive rhodamine B isothiocyanate (RBIRC) onto the inner surface of TMV. The fluorescence intensity ratio of FITC to RBIRC excited at 488 nm responds specifically towards pH value over other interferential agents. By taking use of this single-wavelength excited ratiometric pH probe and confocal laser scanning microscopy, it is shown that the endocytosed TMV is located in a pH decreasing microenvironment and eventually enters lysosomes. This work may provide important guidance on construction of TMV-based nano carriers.

Keywords Tobacco mosaic virus; Ratiometric fluorescence pH probe; Intracellular trafficking

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INTRODUCTION

Viral nanoparticles and virus-like nanoparticles have advantages of monodisperse size, chemically or genetically modifiable surface, high transfection efficiency, and thus exhibit enormous potentials in biomedical field.^[1–3] As a typical rod-like plant virus, tobacco mosaic virus (TMV) is 300 nm in length, 18 nm in outer diameter and has an inner cavity of 4 nm.^[4–6] It is composed of 2130 identical coat proteins self-assembling helically around single-stranded RNA.^[7,8] Due to the high aspect ratio, easy chemical modification, and biocompatibility,^[9–12] TMV is a competitive candidate in diagnosis and drug delivery.^[13–15] For example, Steinmetz's group prepared VCAM-1 peptide-decorated TMV, and loaded both near-infrared dyes and Gd ion in its cavity. This TMV-based nanoparticle performed a dual-modal magnetic resonance and fluorescence imaging of atherosclerotic plaques in mice.^[16] Through chemical conjugation, our group functionalized TMV outer surface with

oligosaccharide, tumor-targeted peptide, or cell-penetrating peptide, which showed great potential in anti-cancer drug delivery and gene delivery.^[15,17,18]

As a nano drug carrier, TMV's intracellular trafficking plays a key role in its cytotoxicity, targeting site, and delivery efficiency. Thus, elucidating the intracellular trafficking route of TMV will be advantageous to designing new drug delivery systems with different targets. In a mammalian cell, to accomplish the physiological processes such as metabolism, the pH value of different organelles is strictly regulated.^[19,20] For example, the early endosomes and late endosomes have pH value of 6.3 and 5.5, respectively. And the pH value of lysosome is as low as about 4.7.^[21,22] Therefore, intracellular trafficking pathway of TMV can be judged by detecting the pH value of its microenvironment. Ratiometric fluorescence pH probe with single-wavelength excited mode is a non-invasive, real-time imaging technique with high spatial-temporal resolution. In addition, it can reduce interference from probe concentration and instrument parameters. Compared with multiple-excitations mode, the single-wavelength excited mode can avoid complicated operation and background noise. As a result, ratiometric fluorescence pH probe with single-wavelength excited mode is a valuable technique in pH sens-

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ing of live cells.^[23–25]

Herein, we reported a single-wavelength excited ratiometric fluorescence pH probe to detect the intracellular trafficking pathway of TMV. Fluorescein isothiocyanate (FITC) and rhodamine B isothiocyanate (RBITC) were selected due to their biocompatibility and high photobleaching resistance.^[26–28] FITC is a pH-sensitive fluorescent dye whose fluorescence intensity can be dramatically enhanced with the increase of pH. RBITC is a pH-insensitive fluorescence dye, whose fluorescence intensity is hardly changed toward different pH values. At an excitation wavelength of 488 nm, FITC and RBITC exhibited emission peaks at 515 and 575 nm, respectively. By conjugating both FITC and RBITC onto TMV, the pH-insensitive fluorescence of RBITC at 575 nm acted as an internal standard, and the fluorescence of FITC at 515 nm increased with pH value, forming a ratiometric fluorescence pH probe (TMV-F&RB). To keep the surface properties of the probe consistent with wild TMV, FITC and RBITC were conjugated onto the inner surface of TMV. According to the fluorescence intensity ratio of FITC and RBITC, intracellular trafficking and distribution of TMV could be imaged. This work can provide guidance for TMV applications in disease diagnosis and treatment.

EXPERIMENTAL

Materials and Instruments

All reagents were used as received without further purification. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC), hydroxybenzotriazole (HOBT), dimethylsulfoxide (DMSO), hydrogen peroxide (H₂O₂), *N*-ethylmaleimide (NEM), and *N*-acetylcysteine (NAC) were purchased from Aladdin Industrial Corporation. Fluorescein isothiocyanate (FITC) was purchased from Acros Organics Corporation. Rhodamine B isothiocyanate (RBITC) was purchased from Sigma-Aldrich Corporation. Sodium hydrate (NaOH), ethylenediamine, sodium carbonate (Na₂CO₃), sodium bicarbonate (NaHCO₃), potassium dihydrogen phosphate (KH₂PO₄), and dipotassium hydrogen phosphate trihydrate (K₂HPO₄·3H₂O) were purchased from Beijing Chemical Works. Lowry Protein Assay Kit was purchased from Beijing Solarbio & Technology Co., Ltd. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (P/S), and LysoTracker® Deep Red were purchased from Life Technologies. Cell Counting Kit-8 (cck-8) was purchased from DOJINDO Molecular Technologies.

Transmission electron microscopic (TEM) images were taken on a JEM-2100F (JEOL, Japan) microscope. Absorption spectra were measured on a U-3900 UV-Vis spectrophotometer (Hitachi, Japan). Fluorescence emission spectra were recorded on a F-4600 fluorescence spectrophotometer (Hitachi, Japan). Fluorescence decay curves were recorded on a FLS1000 Edinburgh spectrometer. Intracellular imaging and colocalization experiment was imaged by a Nikon Eclipse Ti confocal laser scanning microscope (CLSM) with a TDKAI HIT live cell imaging system.

Preparation of TMV-F&RB

Conjugate ethylenediamine on the inner surface of TMV

For this reaction, 1 mL of TMV (10 mg/mL in H₂O) and 1 mL of

HEPES buffer (1 mol/L, pH 7.4) were added into 7 mL of H₂O and mixed well. Then, 385 µL of HOBT (20 mg/mL in DMSO) and 173 µL of ethylenediamine (10 mg/mL in H₂O) were added into the solution. After stirring for 30 min, 165 µL of EDC (10 mg/mL in H₂O) was added into the mixture. Two more portions of 165 µL of EDC aqueous solution were added into the mixture after 6 and 18 h. The reaction was performed at 4 °C for 24 h. The product (TMV-NH₂) was purified by dialysis.

Conjugate FITC and RBITC on the inner surface of TMV-NH₂

For this reaction, 40 µL of Na₂CO₃-NaHCO₃ buffer (1 mol/L, pH 9.0) and 36 µL of DMSO were added into 100 µL of H₂O. After cooling, 200 µL of TMV-NH₂ (2 mg/mL in H₂O), 20 µL of RBITC (2 mg/mL in DMSO), and 4 µL of FITC (2 mg/mL in DMSO) were added into the solution. The reaction was performed at 4 °C for 24 h and protected from light. The product (TMV-F&RB) was purified by dialysis.

Cell Culture

HeLa cells (human cervical cancer epithelial cells) were incubated in DMEM medium with 1% P/S and 10% FBS at 37 °C in a humidified atmosphere containing 5% CO₂.

Cytotoxicity Assay

HeLa cells were seeded in 96-well plates at a density of 8000 cells per well. After culturing overnight at 37 °C and 5% CO₂, the medium was replaced by fresh medium containing TMV-F&RB with different concentrations. After incubation for 24 h, the medium was replaced by fresh medium containing cck-8. After 2–3 h of incubation, the absorbance at 450 nm was recorded by an EnSpire Multimode Plate Reader (PerkinElmer). Cell viability was calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{I_{\text{sample}}}{I_{\text{control}}} \times 100\%$$

where I_{sample} and I_{control} represent the absorbance at 450 nm of cells treated with TMV-F&RB and without any treatment, respectively.

Intracellular Imaging

2×10^5 HeLa cells were cultured overnight in glass bottom dishes. The adherent cells were incubated with TMV-F&RB (0.4 µg/mL) for determined time, such as 0, 1, 4, 7, and 24 h. After changing the growth medium to a new one, the cells were imaged directly on CLSM with a live cell imaging system.

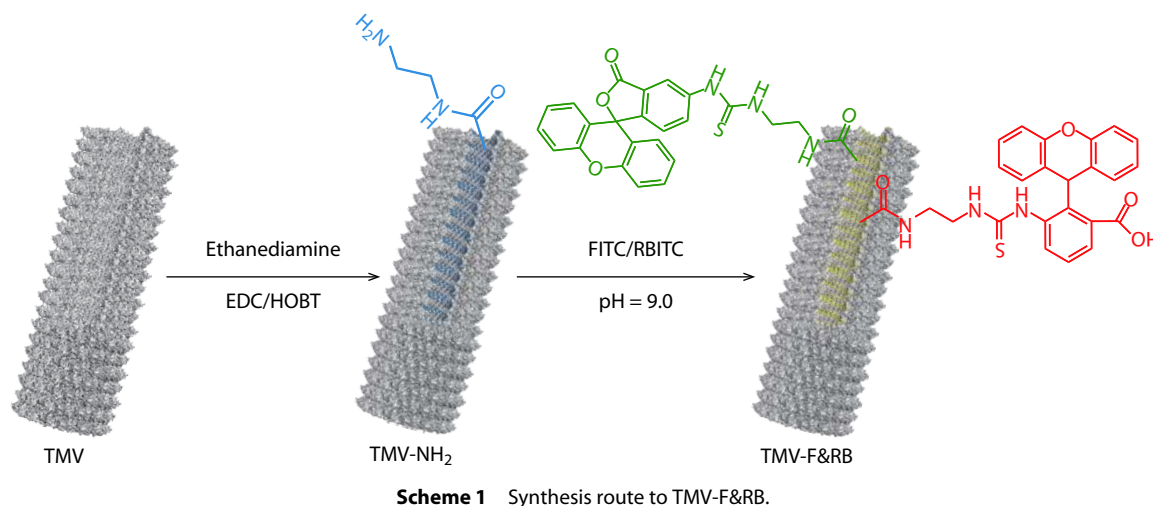
Colocalization Experiment

2×10^5 HeLa cells were cultured overnight in glass bottom dishes. TMV-F&RB were added into cells at a final concentration of 0.4 µg/mL and incubated for 24 h. The culture medium was changed to a new one containing 50 nmol/L LysoTracker® Deep Red and incubated for 30 min. The resulting cells were observed by CLSM with a live cell imaging system.

RESULTS AND DISCUSSION

TMV-F&RB Synthesis and Characterization

TMV-F&RB was obtained by conjugating FITC and RBITC onto the inner surface of TMV. There are two chemical reaction sites on the inner surface of TMV for each coat protein: glutamic acid 97 residue (97 Glu) and glutamic 106 residue (106 Glu).^[11] TMV-F&RB was fabricated as depicted in Scheme 1. (1) Amino groups were introduced on the inner surface of TMV through



conjugation between carboxyl groups of 97&106 Glu and amino groups of ethylenediamine. The obtained product is referred to as TMV-NH₂. (2) TMV-F&RB was obtained by conjugation between amino groups of TMV-NH₂ and isothiocyanate groups of FITC and RBITC. The molar ratio of FITC to RBITC was 1:5. From TEM observation, TMV-F&RB maintains a rod-like structure after reaction which is similar to the structure of TMV (Fig. 1a).

From the UV-Vis absorption spectra, characteristic absorption peak of TMV is 260 nm, while TMV-F&RB has another absorption peak at 560 nm, which is the characteristic absorption peak of RBITC (Fig. 1b). Because FITC exhibits quite low absorbance in aqueous solution, the characteristic absorption for FITC at 488 nm could not be distinguished from the UV-Vis absorption spectrum of TMV-F&RB.^[26] According to SDS-PAGE, the lane of TMV-F&RB emits yellow fluorescence under UV light irradiation, which is a mixture fluorescence of FITC (green) and RBITC (red) (Fig. 1c). As a comparison, the lane of TMV modified with FITC (TMV-FITC) emits green fluorescence, and that of TMV modified with RBITC (TMV-RBITC) emits red fluorescence. These results demonstrate that FITC

and RBITC were successfully grafted onto the inner surface of TMV. After determining the concentration of TMV by Lowry Protein Assay Kit and the concentration of dyes by UV-Vis spectra, there were about 1267 FITC molecules and 716 RBITC molecules conjugated on each virus.

pH Detection Property of TMV-F&RB

After successfully grafting FITC and RBITC on the inner surface of TMV, fluorescence emission spectra (excited at 488 nm) of the product were investigated in 10 mmol/L KH₂PO₄-K₂HPO₄ buffer solutions at different pH values. As shown in Fig. 2(a), the fluorescence intensity of FITC (*I*_{515 nm}) was sequentially enhanced with increasing pH value, while the fluorescence intensity of RBITC (*I*_{575 nm}) changed slightly. More importantly, the fluorescence intensity ratio of FITC and RBITC ($R = I_{515 \text{ nm}}/I_{575 \text{ nm}}$) had a good linear relationship with the pH value. Fig. 2(b) depicts its pH calibration curve, which presents a good linear correlation coefficient ($R^2 = 0.9910$) over the pH range of 5.7–7.4. Fluorescence decay curves of TMV-F&RB were measured in 10 mmol/L K₂HPO₄-KH₂PO₄ buffer at different pH values to demonstrate the fluorescence lifetime. As shown in Figs. 2(c)

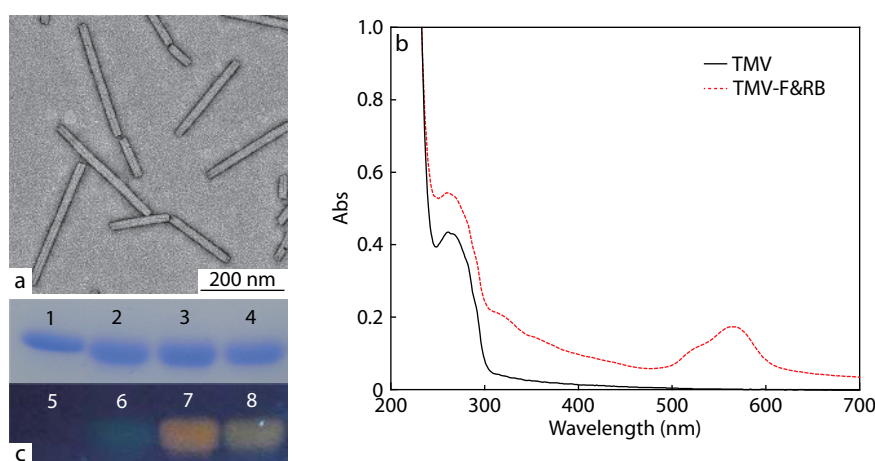


Fig. 1 (a) TEM image for TMV-F&RB. (b) UV-Vis absorption spectra of TMV (black line) and TMV-F&RB (red line). (c) SDS-PAGE analysis of TMV (lanes 1 and 5), TMV-FITC (lanes 2 and 6), TMV-RBITC (lanes 3 and 7), and TMV-F&RB (lanes 4 and 8). Lanes 1–4 are observed by Coomassie Brilliant Blue staining. Lanes 5–8 are fluorescence band excited with UV lamp.

and 2(d), fluorescence lifetimes at 515 and 575 nm were hardly changed with pH values.

The interference of probe concentration and some reactive oxygen species on pH detection was further investigated in 10 mmol/L pH 6.5 $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ buffer. As shown in Fig. 3(a), the fluorescence intensity ratio showed no noticeable change at different probe concentrations. Oxidative-

stress-associated redox chemicals, such as hydrogen peroxide (H_2O_2), glutathione precursor *N*-ethylmaleimide (NEM), and glutathione inhibitor *N*-acetylcysteine (NAC), had no obvious effect on the fluorescence intensity ratio (Fig. 3b) either. These results indicate that TMV-F&RB can be used as a ratiometric fluorescence pH probe to detect intracellular trafficking and subcellular organelle distribution of TMV.

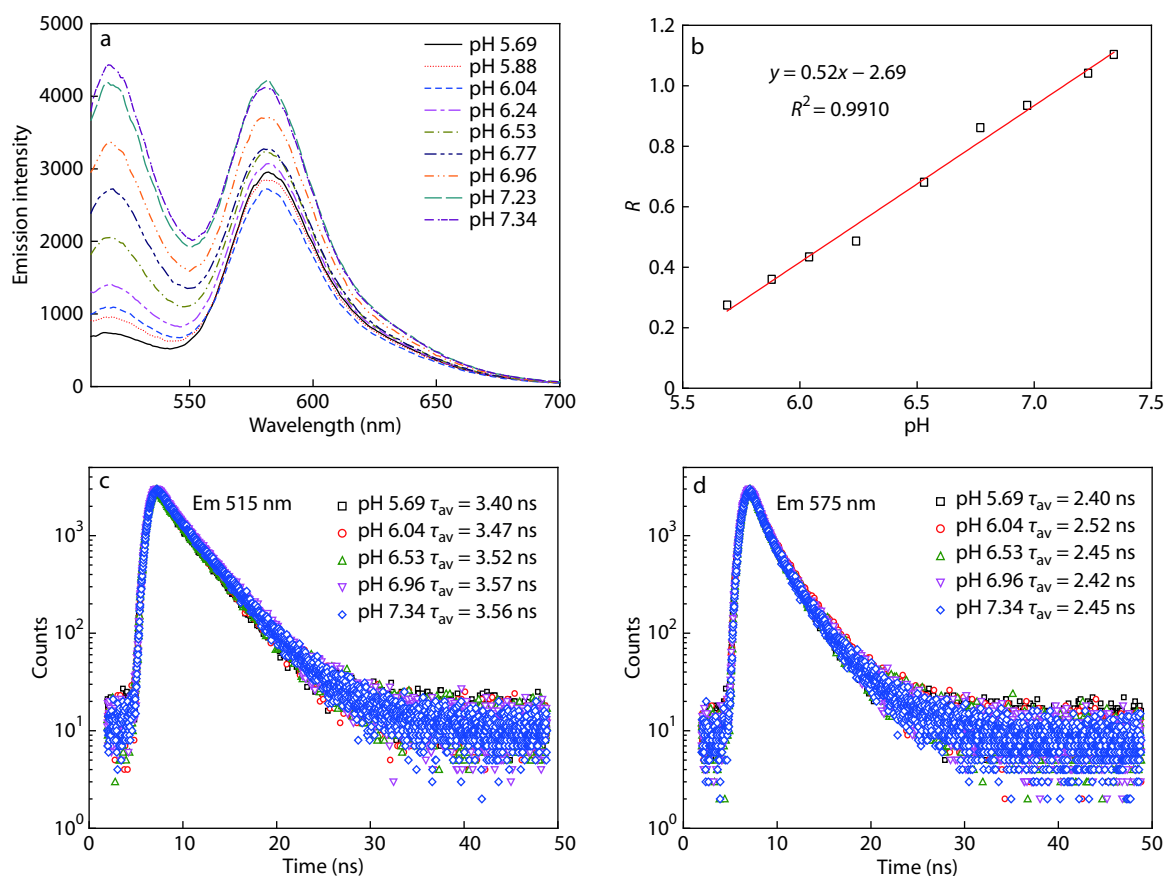


Fig. 2 (a) Fluorescence emission spectra of 0.05 mg/mL TMV-F&RB excited at 488 nm in 10 mmol/L $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ buffer at different pH values. (b) Linear relationship between R and pH in the range of 5.7–7.4. (c, d) Fluorescence decay curves of TMV-F&RB in 10 mmol/L $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ buffer at different pH values at monitoring wavelengths of (c) 515 nm and (d) 575 nm. Average fluorescence lifetime (τ_{av}) was marked. $\lambda_{ex} = 450$ nm.

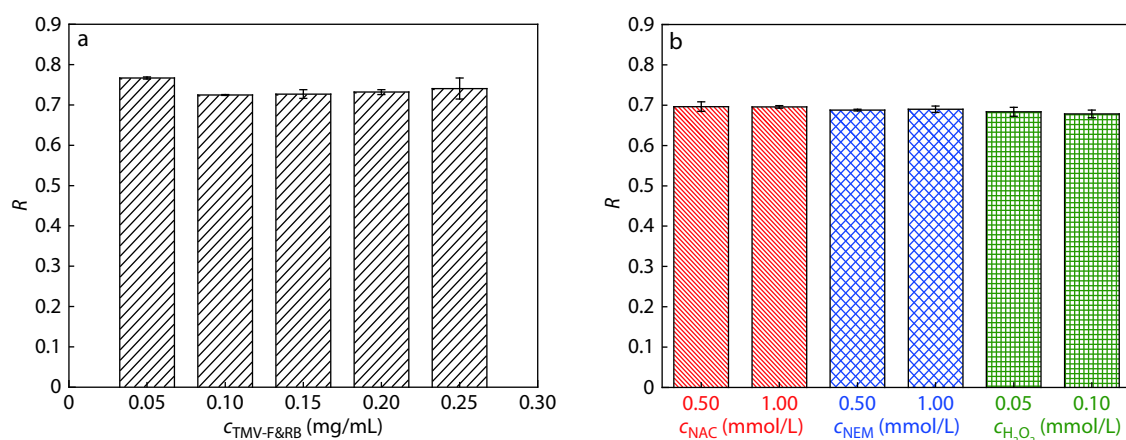


Fig. 3 (a) R values of TMV-F&RB at different TMV-F&RB concentrations in 10 mmol/L pH 6.5 $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ buffer. (b) R values of 0.05 mg/mL TMV-F&RB in 10 mmol/L pH 6.5 $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ buffer in the presence of NAC, NEM, or H_2O_2 at different concentrations.

Intracellular Imaging

Cytotoxicity of TMV-F&RB was explored prior to intracellular imaging experiments. After being incubated with TMV-F&RB for 24 h at a concentration ≤ 100 $\mu\text{g/mL}$, cell viability of HeLa cells was not significantly decreased, confirming low cytotoxicity of TMV-F&RB (Fig. 4). To investigate the intracellular trafficking of TMV, TMV-F&RB was co-cultured with HeLa cells. The obtained cells were observed by confocal laser scanning microscopy (CLSM). As shown in Fig. 5, when TMV-F&RB was just added into the cell culture medium, fluorescence was formed in the cells from FITC channel and RBITC channel. This phenomenon indicates that TMV can enter HeLa cells quickly. As co-culture time prolonged, the fluorescence intensity from the FITC channel (green) gradually increased, whereas that from the RBITC channel (red) hardly changed. The ratio images from the FITC channel and RBITC channel exhibited a distinct time dependent pattern. As the culture time increased, the color of the ratio channel changed from green to blue, indicating that the pH value of TMV microenvironment was gradually decreasing (see the color bar obtained from TMV-F&RB solutions in standard buffers). When foreign substances enter the cells through endocytosis, they will firstly enter the early endosomes. Then the early endosomes mature into late endosomes, and finally form lysosomes. Depending on the pH of organelles, this process is usually accompanied with pH decreasing, which is consistent with the imaging results. These results demonstrate that TMV-F&RB successfully imaged the intracellular trafficking pathway of endocytosed TMV.

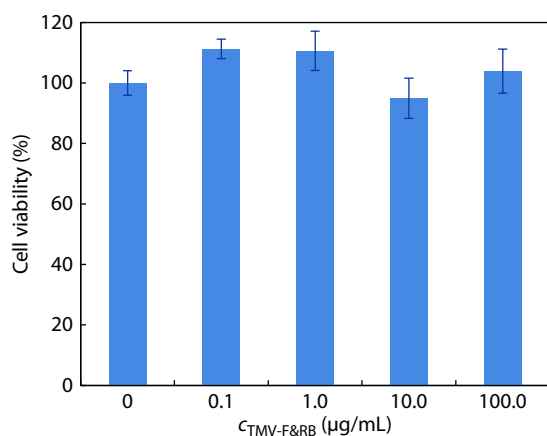


Fig. 4 Cell viability of HeLa cells incubated with TMV-F&RB at different concentrations for 24 h. Data are shown as mean value $\pm$ S.D. ($n = 3$). Error bars represent the standard deviation of three replicates.

To investigate the intracellular distribution of TMV-F&RB, colocalization experiments were performed (Fig. 6). HeLa cells were incubated with TMV-F&RB for 24 h, followed by staining with 50 nmol/L LysoTracker® Deep Red for another 30 min to label lysosomes. Two color channels were used for CLSM observation: RBITC channel (570–610 nm) and LysoTracker channel (650–690 nm, pseudo-colored as green for clarity). Convincing yellow color from merge channel confirms that the red fluorescence from TMV-F&RB could overlap with the green fluorescence from LysoTracker® Deep Red. Pearson's

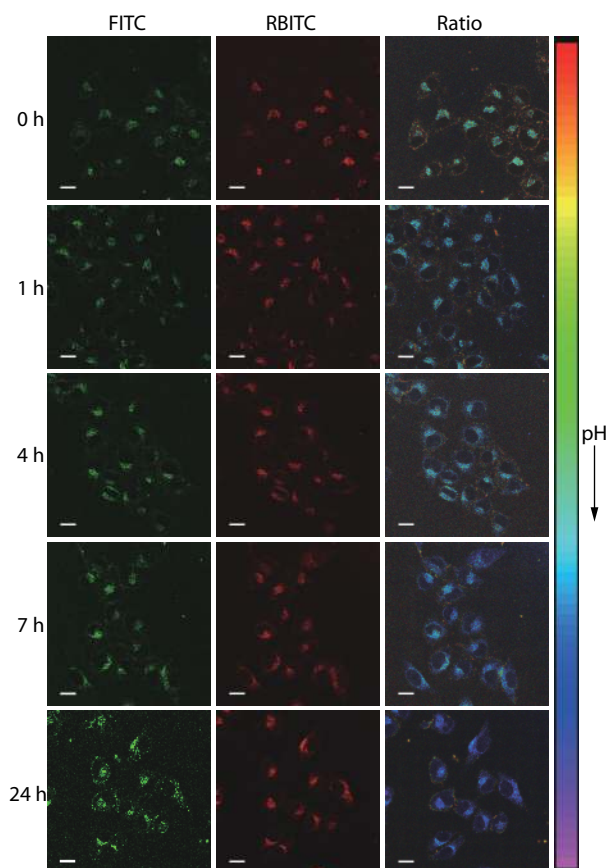


Fig. 5 CLSM images of HeLa cells incubated with TMV-F&RB for 0, 1, 4, 7, and 24 h. The first and second columns are FITC channel and RBITC channel collected with $\lambda_{\text{em}} = 510\text{--}550$ nm and $\lambda_{\text{em}} = 570\text{--}610$ nm, respectively. Ratio channel is the fluorescence intensity ratio between FITC and RBITC channel. Scale bar is 20 μm .

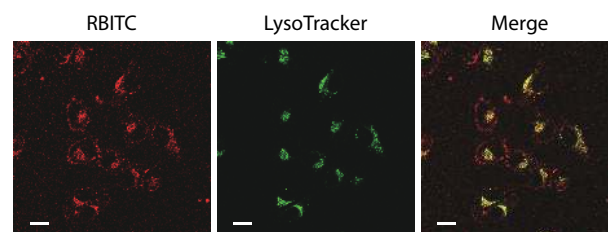


Fig. 6 CLSM images of HeLa cells co-stained with TMV-F&RB and LysoTracker® Deep Red. RBITC channel and LysoTracker channel were collected with $\lambda_{\text{em}} = 570\text{--}610$ nm and $\lambda_{\text{em}} = 650\text{--}690$ nm, respectively. Merge channel was obtained from RBITC and LysoTracker channel. Scale bar is 20 μm .

correlation coefficient between RBITC channel (TMV-F&RB) and LysoTracker channel (LysoTracker) is 0.70. The results confirm that TMV-F&RB eventually entered lysosomes.

CONCLUSIONS

In this work, we have successfully prepared a ratiometric fluorescent pH probe, TMV-F&RB, to image intracellular trafficking and subcellular organelle dispersion of TMV. TMV-F&RB was prepared by conjugating pH-sensitive FITC and pH-

insensitive RBITC onto the inner surface of TMV. Fluorescence intensity ratio of FITC and RBITC had good linear relationship with pH value in the range of 5.7 to 7.4, and was not affected by interferential agents such as probe concentration and oxidative-stress-associated redox chemicals. Intracellular imaging confirmed that pH value of the microenvironment surrounding TMV gradually decreased, which is consistent with the intracellular trafficking pathway after endocytosis. Colocalization experiments confirmed that TMV eventually entered the lysosomes.

ACKNOWLEDGMENTS

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