**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.\(^1\) 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.\(^2\) It is composed of 2130 identical coat proteins self-assembling helically around single-stranded RNA.\(^3\)\(^,\)\(^4\) Due to the high aspect ratio, easy chemical modification, and biocompatibility,\(^5\)\(^-\)\(^9\)\(^,\)\(^10\) TMV is a competitive candidate in diagnosis and drug delivery.\(^11\)\(^-\)\(^13\) 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.\(^14\) 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-
ing of live cells.\textsuperscript{22--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 photo-bleaching resistance.\textsuperscript{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-piperazinethanesulfonic acid (HEPES), 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC), dimethyl sulfoxide (DMSO), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), N-ethylmaleimide (NEM), and N-acetyl cysteine (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 (Na\textsubscript{2}CO\textsubscript{3}), sodium bicarbonate (NaHCO\textsubscript{3}), potassium dihydrogen phosphate (KH\textsubscript{2}PO\textsubscript{4}), and dipotassium hydrogen phosphate trihydrate (K\textsubscript{2}HPO\textsubscript{4}·3H\textsubscript{2}O) 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® (P/S), and LysoTracker® (P/S) were selected due to their biocompatibility and high photobleaching resistance.

Transmission electron microscope (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 Edinburge spectrometer. Intracellular imaging and colocalization experiment was imaging 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 H\textsubscript{2}O) and 1 mL of HEPES buffer (1 mol/L, pH 7.4) were added into 7 mL of H\textsubscript{2}O and mixed well. Then, 385 μL of HOBT (20 mg/mL in DMSO) and 173 μL of ethylenediamine (10 mg/mL in H\textsubscript{2}O) were added into the solution. After stirring for 30 min, 165 μL of EDC (10 mg/mL in H\textsubscript{2}O) 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-NH\textsubscript{2}) was purified by dialysis.

**Conjugate FITC and RBITC on the inner surface of TMV-NH\textsubscript{2}**

For this reaction, 40 μL of Na\textsubscript{2}CO\textsubscript{3}-NaHCO\textsubscript{3} buffer (1 mol/L, pH 9.0) and 36 μL of DMSO were added into 100 μL of H\textsubscript{2}O. After cooling, 200 μL of TMV-NH\textsubscript{2}, 2 (mg/mL in H\textsubscript{2}O), 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% CO\textsubscript{2}.

**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% CO\textsubscript{2}, 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 \(I_{\text{sample}}\) and \(I_{\text{control}}\) represent the absorbance at 450 nm of cells treated with TMV-F&RB and without any treatment, respectively.

**Intracellular Imaging**

2 × 10\textsuperscript{4} 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\textsuperscript{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 97 residue (97 Glu) and glutamic 106 residue (106 Glu).\textsuperscript{[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-NH$_2$. (2) TMV-F&RB was obtained by conjugation between amino groups of TMV-NH$_2$ 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 KH$_2$PO$_4$-K$_2$HPO$_4$ buffer solutions at different pH values. As shown in Fig. 2(a), the fluorescence intensity of FITC ($I_{515 \text{ nm}}$) was sequentially enhanced with increasing pH value, while the fluorescence intensity of RBITC ($I_{575 \text{ nm}}$) 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 K$_2$HPO$_4$-KH$_2$PO$_4$ buffer at different pH values to demonstrate the fluorescence lifetime. As shown in Figs. 2(c)

![Scheme 1](image-url)  
**Scheme 1** Synthesis route to TMV-F&RB.
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 KH$_2$PO$_4$-K$_2$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$_2$O$_2$), glutathione precursor N-ethylmaleimide (NEM), and glutathione inhibitor N-acetylcycteine (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.
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 μg/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.

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 RBIC onto the inner surface of TMV. Fluorescence intensity ratio of FITC and RBIC 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.

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