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Selective labeling and monitoring pH changes of lysosomes in living cells with fluorogenic pH sensors

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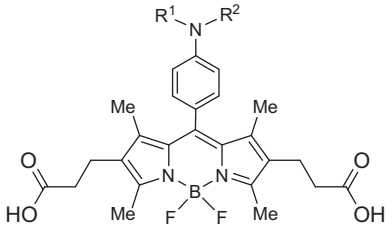
ABSTRACT

New BODIPY-based pH probes have been designed with excitation and emission wavelengths suitable for fluorescence microscopy and flow cytometry. These pH probes are cell-permeable, selectively label lysosomes, and can be used for noninvasive monitoring of lysosomal pH changes during physiological and pathological processes.

Intracellular pH plays many important roles in cell functions, such as cell proliferation[1] and apoptosis.[2–5] Monitoring pH changes inside living cells is also critical for studying cellular internalization pathways, including phagocytosis,[6] endocytosis,[7,8] and receptor ligand internalization.[9] Abnormal intracellular pH values are often associated with some common diseases such as cancer[10] and Alzheimer's disease.[11] Acidic organelles, for example, lysosomes (pH 4.5–5.5)[12] and endosomes (pH 5.4–6.2),[13] participate in the critical functions of endocytic and digestive processes.

Fluorescent indicators for monitoring intracellular pH changes in different organelles can be used to investigate the role of intracellular pH in diverse physiological and pathological processes, including cell proliferation, apoptosis, and endocytosis.[14] The pH inside a cell varies by only fractions of a pH unit, and such changes may be quite slow. Different pH indicators with various pK_a values are necessary to monitor different pH environments in cells. Many methods based on pH probes have been reported to detect pH changes in acidic organelles and to follow trafficking through acidic organelles.[15–21]

Recently, Urano et al. described BODIPY-based pH-activatable probes with tunable pK_a ranging from 3.8 to 6.0, and used pH-activatable probe-antibody conjugates for in vivo imaging of cancer cells in mice.[22] In that work four different BODIPY fluorogenic pH sensor molecules, compounds **1a–d**, were prepared, with hydrogen, methyl or ethyl substituents on the aniline nitrogen, to study the effect of nitrogen substituents on pK_a and cellular analysis properties. Inspired by that work, we have extended those insights to design new cell-permeable BODIPY analogs for live cell imaging.



1a R^1, R^2 = H
1b R^1, R^2 = CH_3
1c R^1 = CH_3, R^2 = CH_2CH_3
1d R^1, R^2 = CH_2CH_3

Scheme 1 shows the synthesis of the new BODIPY-based pH probes **4a–d**. Each pyrrole (**2a, b**) was treated sequentially with a benzaldehyde (**3a–c)** in the presence of a catalytic amount of TFA,[23] *p*-chloranil, then TEA and $BF_3{\cdot}OEt_2$ to afford BODIPY-based pH probes **4a–d** with 20%, 26%, 27%, and 53% yield, respectively. The fluorescence emission intensities of the resulting new probes **4a–d** were measured at pH ranging from 2.0 to 8.0. These results are shown in the fluorescence emission spectra of probes **4a–d** in Figure 1. Importantly, these probes are almost non-fluorescent (fluorescence quantum yield (Φ_{fl}) <0.001) at neutral pH 7.0, due

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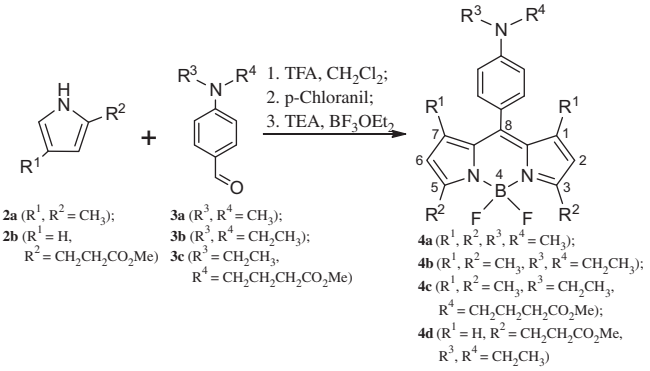

2a (R^1, R^2 = CH_3);
2b (R^1 = H, R^2 = $CH_2CH_2CO_2Me$)

3a (R^3, R^4 = CH_3);
3b (R^3, R^4 = CH_2CH_3);
3c (R^3 = CH_2CH_3, R^4 = $CH_2CH_2CH_2CO_2Me$)

4a (R^1, R^2, R^3, R^4 = CH_3);
4b (R^1, R^2 = CH_3, R^3, R^4 = CH_2CH_3);
4c (R^1, R^2 = CH_3, R^3 = CH_2CH_3, R^4 = $CH_2CH_2CH_2CO_2Me$);
4d (R^1 = H, R^2 = $CH_2CH_2CO_2Me$, R^3, R^4 = CH_2CH_3)

Scheme 1. Synthesis of BODIPY-base pH probes **4a–d**.

concentration with Bovine Pulmonary Artery Endothelial (BPAEC) cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) at 37 °C for 30 min. After a simple wash in PBS buffer, fluorescent images were captured on a Zeiss Axioskop 2 fluorescence microscope with 40× objective equipped with a Hamamatsu ORCA-ER CCD camera using a 480 ± 10 nm band-pass excitation filter and a 515 ± 10 nm band-pass emission filter from Omega Optical. All images were collected at 50 ms exposure time and processed with identical leveling. The fluorescent intensities of cell images were analyzed using SlideBook software. The cell images and relative fluorescence intensities are shown in Figure 2. Our results clearly show that these pH probes **4a–d** are selectively labeling the acidic organelle lysosomes, and they become highly fluorescent under the conditions in the acidic lysosomes. The fluorescent intensities vary based on the pK_a of the

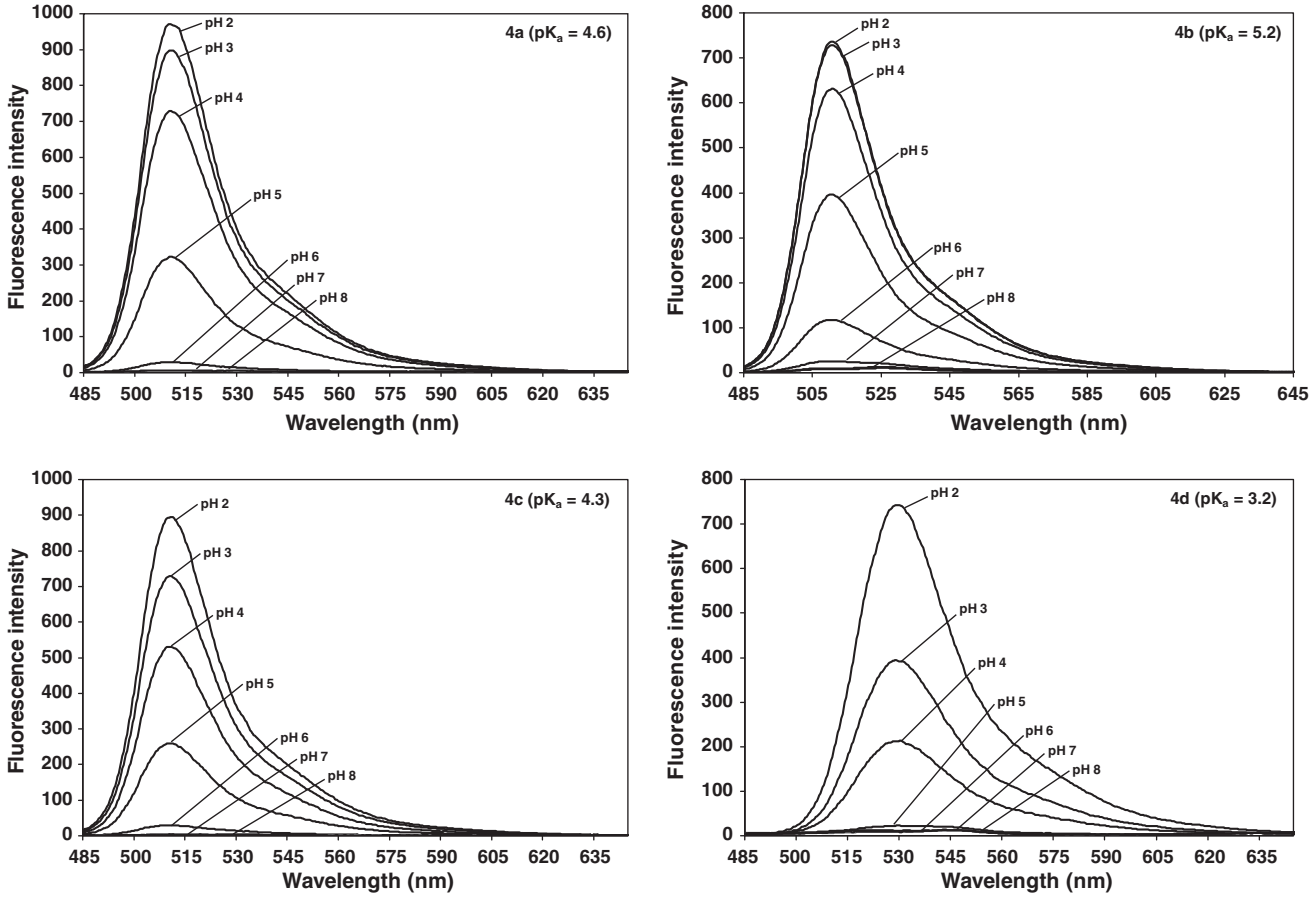

Figure 1. Fluorescence emission spectra (λ_{ex} = 480 nm) of probes **4a–d** as a function of pH. Probes **4a–d** were prepared at 100 nM concentration at vary pH buffer. 100 mM NH_4OAc was used to make pH 2–4 buffers; PBS was used to make pH 5–8 buffers.

to photo-induced electron transfer (PeT)[24] from the aniline moiety to the BODIPY fluorophore, but they become highly fluorescent at acidic pH ~2.0 (fluorescence quantum yield Φ_{fl} of probe **4a–d** is 0.52, 0.50, 0.49, and 0.45, respectively). The emission maximum of probe **4d** is red-shifted by 20 nm in comparison to those of probes **4a–c**. By changing the substitution of the *N*-alkyl group on the aniline moiety and the substituent on the BODIPY fluorophore, a series of pH probes with tunable pK_a values ranging from 3.2 to 5.2 have been developed.

To demonstrate the selective labeling of lysosomes in live cells, we incubated the BODIPY-based pH probes **4a–d** at 1 μM pH probes, with highest fluorescent intensity for probe **4b** with pK_a = 5.2, and lowest fluorescent intensity for probe **4d** with pK_a = 3.2.

One good property of these pH probes is the sharp change in emission intensity from pH 3.0 to 6.0. This provides a good opportunity to use these pH probes to monitor the pH changes in the lysosomes during physiological and pathological processes. To demonstrate this application, the BPAEC cells in DMEM media supplemented with 20% FBS were treated with an antimalarial drug, chloroquine, at 100 μM concentration at 37 °C for 30 min. This drug is known to induce a lysosomal pH increase.[19] Probe **4b**

(1 μM) and a nuclear stain dye (Hoechst 33342, 1 μg/mL) were incubated with chloroquine treated and untreated BPAEC cells in DMEM media supplemented with 20% FBS at 37 °C for 30 min, respectively. The cell images were recorded by fluorescence microscopy (as described above). The nuclear stain Hoechst 33342 was imaged using a 365 ± 5 nm band-pass filter for excitation and a 400 ± 5 nm cutoff filter for emission. The BODIPY fluorophore was imaged using a 480 ± 10 nm band-pass filter for excitation and a 515 ± 10 nm band-pass filter for emission. Exposure times of 50 and 100 ms, for the nuclear stain and BODIPY dye channels, respectively, were used for image collection. All images were processed with identical leveling, and the fluorescent signals were analyzed using SlideBook software. As shown in Figure 3, the fluorescent signals in the lysosomes of chloroquine treated cells decreased ~80%, compared to untreated cells using probe **4b**. This demonstrates that probe **4b** is highly sensitive to pH change in the lysosomes. The change in fluorescent intensity is consistent with the lysosomal pH change from pH ~4.5 to ~6.5 after chloroquine treatment.[25] The high sensitivity response to the lysosomal pH change is an important application of probe **4b** in noninvasive monitoring of lysosomal pH changes.

In summary, we have prepared a series of BODIPY-based pH probes **4a–d** with tunable pK_a ranging from 3.2 to 5.2. These pH probes are not fluorescent at neutral pH and highly fluorescent at low pH ~4. The BODIPY-based pH probes **4a–d** have excitation wavelength at ~488 nm and emission wavelength at ~510–530 nm, and are suitable for fluorescence microscopy and flow cytometry. In addition, these pH probes are cell-permeable and selectively label lysosomes. New pH probes **4a–d** are useful for lysosome labeling, and for noninvasive monitoring of lysosomal pH changes during physiological and pathological processes.

Supplementary data

Supplementary data (experimental procedures, structure characterization, and optical properties for BODIPY-based pH probes **4a–d**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.137.

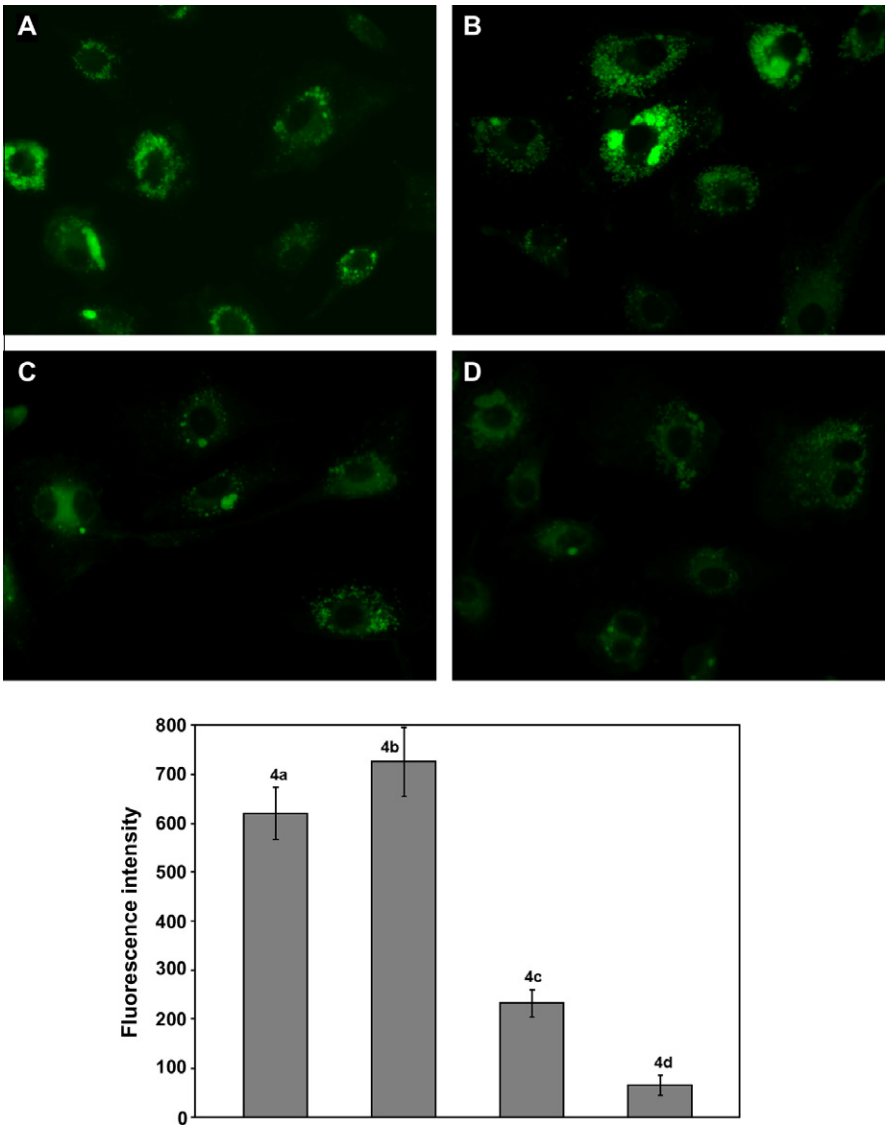

Figure 2. The cell images and relative fluorescence intensities of pH probes **4a–d**. Cell imaging labeled with probes **4a–d** are shown in A, B, C, and D, respectively. The fluorescence intensities were averaged in triplicate.

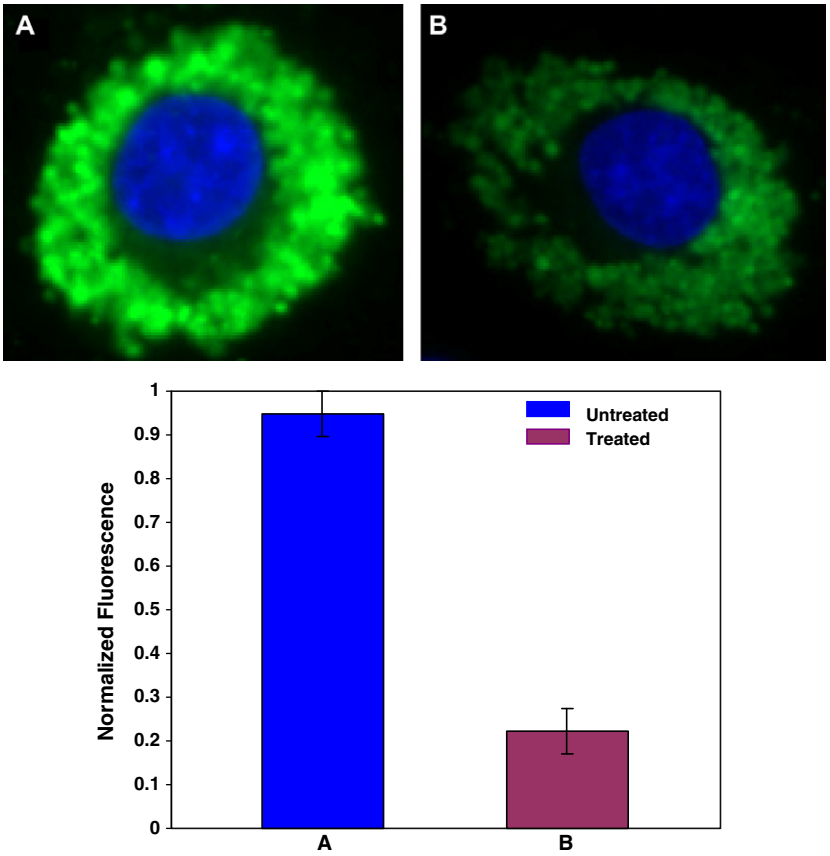

Figure 3. Monitoring lysosomal pH change induced with chloroquine. Untreated cells incubated with probe **4b** colored in green (A), treated cells incubated with probe **4b** colored in green (B). Fluorescence signal from the nuclear stain, Hoechst 33342, colored in blue. The normalized fluorescence values were averaged in triplicate.

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