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Near-infrared Fluorescent Probes with Higher Quantum Yields and Neutral pKa Values for the Evaluation of Intracellular pH

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We have developed two near-infrared fluorescent probes for pH, named pH-A and pH-B. The fluorescence quantum yields of pH-A and pH-B are 0.19, 0.095 in methanol. And there is the neutral pKa value of pH-A (pKa=7.2). What is the most important is that these probes can stain the cells, which can reflect the changes of pH in cells.

The varieties of intracellular pH play important roles in various biological processes such as receptor-mediated signal transduction, enzymatic activity,[1] cell proliferation,[2] apoptosis,[3] ion transport,[4] and homeostasis.[5] Abnormal pH values are often associated with cell dysfunction and some diseases.[6, 7] Thus, monitoring intracellular pH values can provide direct information of living cells.

Fluorescence probes have been widely studied in the fluorescence imaging in living cells, tissues and organisms.[8-10] In order to search a practicable luminescent probe of pH, it may be examined by spectroscopy and microscopy inside living cells. The most important property for the probe is that it needs to be cell-permeable, kinetically stable and non-toxic in living cells. So far, a number of pH probes have been studied and some are commercially available.[11-13] However, many probes have bad permeability of cell membrane, poor stability, low fluorescence quantum yield, short analytical wavelength or lower or higher pKa than physiological conditions.[12-14] Thus development of new pH probe that has available pKa and long wavelength is still in demand.

Cyanines, a classic type of near-infrared fluorochromes, have been frequently employed to develop different spectroscopic probes for imaging studies,[14-16] but they are known to have poor stability and low fluorescence quantum yield as a result of ready autoxidation and photooxidation. And rhodamine fluorophores, which have high fluorescence quantum yields, excellent biocompatibility and high sensitivity under physiological conditions, have been used extensively to measure intracellular pH values.[17] But they display strong red fluorescence (excitation/emission, 563/572nm) at acidic pH because of opening of its cyclolactam ring but becomes non-fluorescent at basic or neutral pH when the ring is closed.[17, 18] In this context, we want to develop the pH probes, which combine cyanines and rhodamine into one molecule, and which have contrasting fluorescence emission in response to pH changes, and therefore would be a fluorescent probe to measure intracellular pH values.

Tab-1. Photophysical properties of pH-A and pH-B

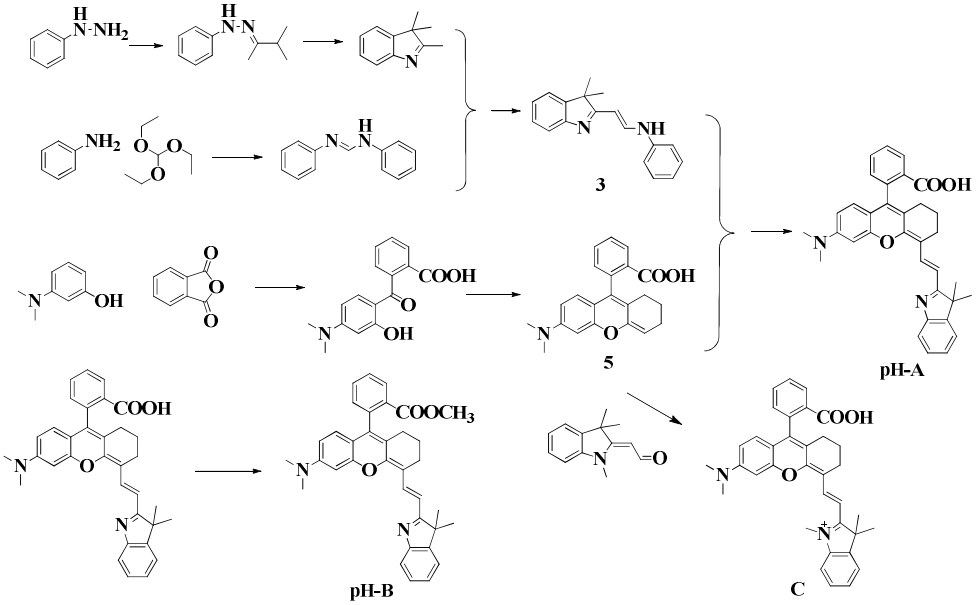
			pH-A	*pH-B*	*C*
CH_3OH	λ_{abs} (nm)		703	721	697
	ε (M^{-1} cm^{-1})		93000	30000	110000
	λ_{em} (nm)		728	738	728
Φ_f	CH_3OH		0.2[a]	0.095	0.41
	CH_3OH : PBS = 1:5	pH=4	0.08	0.04	-
		pH=5	0.09	0.06	-
		pH=6	0.1	0.03	-
		pH=7	0.1	-	-
		pH=8	-	-	-

[a] Reported 3, 5-bi (p–methoxy) phenyl-1, 7-bi (p-bromo) phenyl **aza-BODIPY** (Φ_f 0.42, in toluene) is used as standard

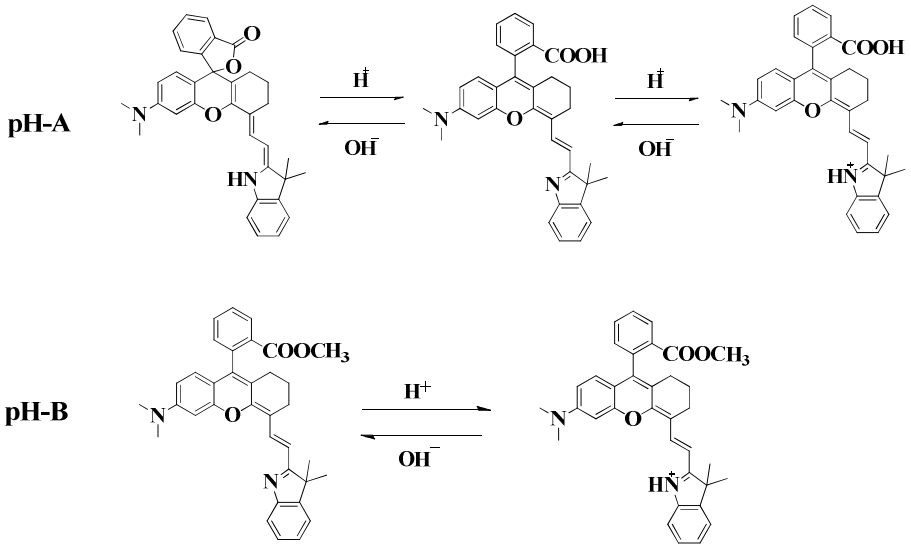
With these criteria in mind, we have looked up the literatures, and studied the properties of some dyes. In 2012, Lin's group presented a strategy to synthesize near-infrared fluorescent dyes.[19] And we have found fluorescent dye **C** has a large absorption extinction coefficient, high fluorescence quantum yield (Φ_f 0.41 in methanol) with near-infrared emission peak at 728 nm, good photostability and chemical stability. Inspired by the attractive photophysical properties of dye **C**, we design and synthesize two near-infrared fluorescent pH probes, named **pH-A** and **pH-B**, which have the similar fluorophoric structure. And we speculate their pKa close to neutral and high fluorescence quantum yield. Fortunately, two near-infrared fluorescent pH probes have been obtained. And the probes have good permeability of cell membrane, long analytical wavelength and available pKa and low toxic in living cells. And as is shown in Tab-1, the fluorescence quantum yields of **pH-A** and **pH-B** are 0.19, 0.095 in methanol. As far as we know, only a few near-infrared pH probes possessing neutral pKa have been reported, and most of them have extremely low fluorescence quantum yields.[12-14] Among them, the highest one is 0.13.[20] Therefore,

compared with the previous NIR neutral-pH probes, the quantum yields of **pH-A** and pH-B are considerable higher.

We have synthesized the probes, named **pH-A and pH-B**, new near-infrared fluorescent probes. The probe, **pH-A**, is synthesized from compound 3 and compound 5 in acetic acid (Scheme 1 and Scheme S1 in the Supporting Information). Compound 5 is synthesized according the literature.[19, 21] The probe, **pH-B**, is obtained from **pH-A** which reacts with $SOCl_2$ in methanol. The **dye-C** is synthesized according the literature.[19] Then the fluorescence quantum yields are measured in methanol. The fluorescence quantum yields are 0.19 for **pH-A**, 0.095 for **pH-B**, 0.4 for **C**. (The standard **aza-BODIPY**).[22]



Scheme 1. Synthesis of **pH-A**, **pH-B** and **dye-C**.



Scheme 2. The structures of **pH-A** and **pH-B** changes with pH.

Spectroscopic studies of probe **pH-A** and **pH-B** in various buffer solutions and different pH values are undertaken using UV/Vis absorption and fluorescence spectroscopy. The probes display sensitive absorption and fluorescence spectroscopic responses to changes in pH values. As shown in Figure 1a, 1b, 1c, and 1d, the probe **pH-A** has the maximum absorption band at 713nm due to **S0→S1** transition, a shoulder peak at 660nm, and strong fluorescence emission at 740nm in pH 6.4 media. Upon excitation at λ=695nm, the near-infrared emission intensity at λ=740nm of **pH-A** has a large decrease with the changes in pH values from 6.4-8.5. And the fluorescence intensity of **pH-A** displays linear responses to pH values in the range from 6.8 to

7.8. The analysis of fluorescence intensity of **pH-A** as a function of pH by using the Henderson-Hasselbach-type mass action equation yields pKa value of 7.2. The absorption of **pH-A** also has a large decrease with the changes in pH from 6.4-8.5 (Figure 1c and 1d).

As shown in Figure 1e, 1f, 1g and 1h, the probe **pH-B** has the maximum absorption band at 745nm due to **S0→S1** transition, a shoulder peak at 690nm, and strong fluorescence emission at 753nm in pH 5.0 media. Upon excitation at λ=705nm, the near-infrared emission intensity at λ=753nm of **pH-B** a large decrease with the changes in pH values from 5.4-7.2. The analysis of fluorescence intensity of the probe **pH-B** as a function of pH by using the Henderson-Hasselbach-type mass action equation yields pKa value of 6.1. And there is a minor change of the absorption with the increase of pH.

There is a large difference of the changes of absorption spectra between the probe **pH-A** and the probe **pH-B** (Figure 1c, 1d, 1g, and 1h), owing to the different effect of pH to the conjugation skeleton structures. In basic solution, **pH-A** exist in a form of cyclolactam leuco (Scheme 2) which possesses a short conjugation length and thus a small absorption coefficient in long wavelength region. With the decrease of pH, the transformation from cyclolactam to the ring-open chromophore takes place, so that the long wavelength absorption increases remarkably. For **pH-B**, it has been esterified, so it cannot form the cyclolactam leuco and exists always in the form of the ring-open chromophoric structure. This is why **pH-B** shows stable absorption spectra without influence by pH changes.

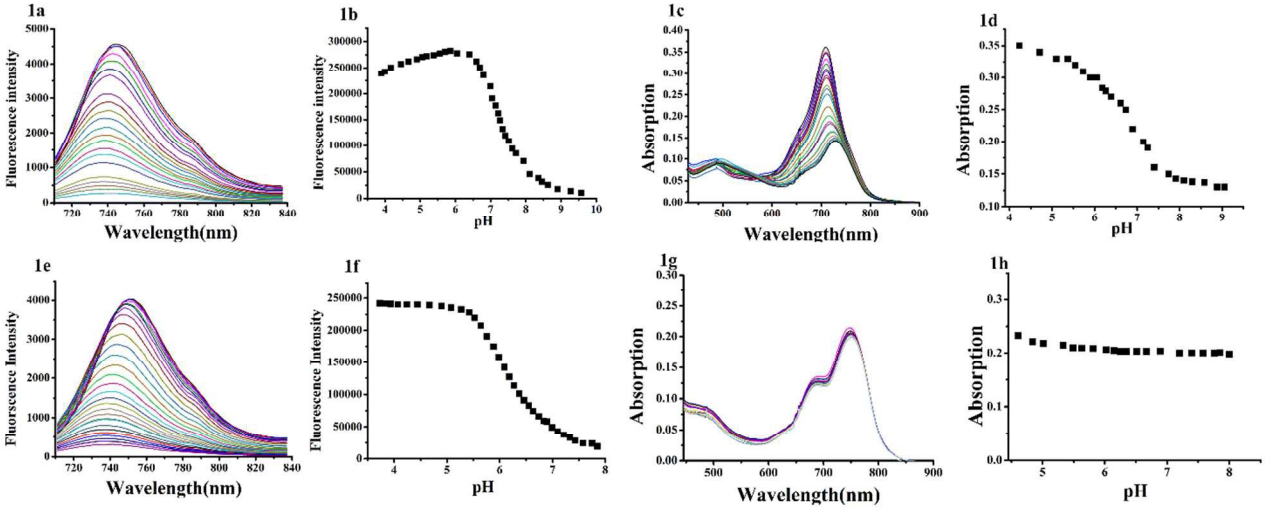


Figure 1. 10 mM fluorescent probe at different pH values in buffer solution containing 20% ethanol. 1a: Fluorescent spectra of **pH-A** at different pH values (pH values from 6.4-8.5); 1b: pH effect on fluorescence intensity of **pH-A** (pH values from 4.0-9.6); 1c: Absorption spectra of **pH-A** at different pH (pH values from 4.2-9.2); 1d: pH effect on absorption of **pH-A** at 713nm (pH values from 4.2-9.2); 1e: Fluorescent spectra of **pH-B** at different pH values (pH values from 5.4-7.5); 1f: pH effect on fluorescence intensity of **pH-B** (pH values from 3.8-7.9); 1g: Absorption spectra of **pH-B** at different pH (pH values from 4.5-8.2); 1h: pH effect on absorption of **pH-B** at 748nm (pH values from 4.5-8.2).

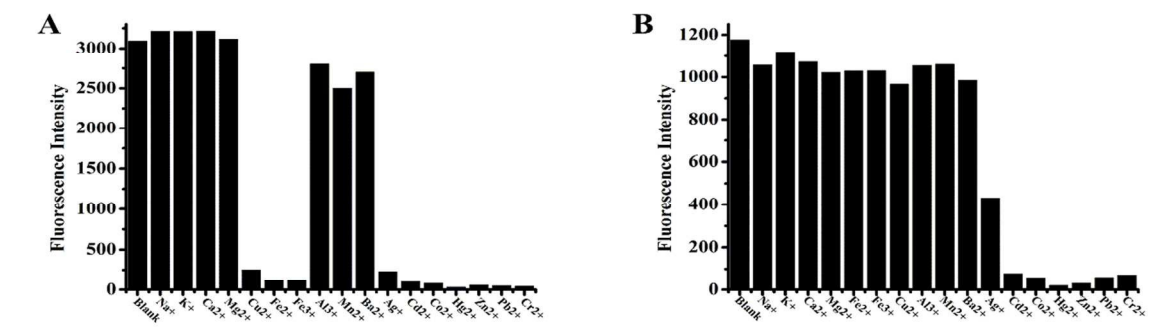


Figure 2. a: Fluorescent responses of 2.5 μM fluorescent probe **pH-A** to pH at 5.8 in the absence and presence of different metal ions (200 μM); b: Fluorescent responses of 2.5 μM fluorescent probe **pH-B** to pH at 4.84 in the absence and presence of different metal ions (200 μM).

To determine any interference on the pH measurement by biological molecules, we measure the fluorescence spectra using probe **pH-A** and **pH-B** in the presence of metal ions (Figure 2: Na^+, K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Mn^{2+}, Ba^{2+}, Ag^+,

Cd^{2+}, Co^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, Cr^{2+}). Fluorescent probe **pH-A** displays no response to 200μM Na^{+}, K^{+}, Ca^{2+}, Zn^{2+}, Al^{3+}, Mn^{2+}, Ba^{2+}. But it responses to 200μM Cu^{2+}, Fe^{2+}, Fe^{3+}, Ag^{+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, Cr^{2+}. Fluorescent probe **pH-B** displays no response to metal ions, such as Na^{+}, K^{+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} Al^{3+}, Mn^{2+} (Figure 2). But it responses to 200μM Ag^{+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, Cr^{2+}.

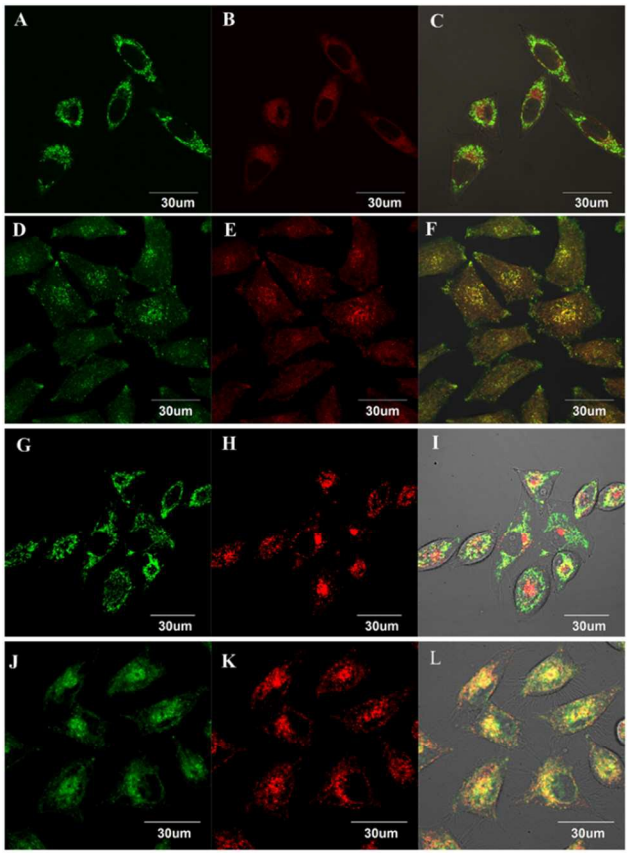


Figure 3. Colocalization imaging studies of **pH-A** and **pH-B** in MCF-7 cells. A and G Rh123 Channel 1: λ_{ex}=488nm, λ_{em}=498-560nm. B and E **pH-A** Channel 2: λ_{ex}=635nm, λ_{em}=655-755nm. D and J DND 189 Channel 1: λ_{ex}=488nm, λ_{em}=498-560nm. H and K **pH-B** Channel 2: λ_{ex}=635nm, λ_{em}=655-755nm. C overlay of A and B (Pearson coefficient 0.67). F overlay of D and E (Pearson coefficient 0.87). I overlay of G and H (Pearson coefficient 0.69). L overlay of J and K (Pearson coefficient 0.95).

To confirm the applicability of **pH-A** and **pH-B** in living cells, we attempt to label MCF-7 cells. As shown in Figure 3 and Figure 4, the probes are easy to stain in MCF-7 cells and to obtain the high resolution fluorescent images. A commercially available mitochondrial dye (Rh123) and a commercially available lysosome dye (DND 189) are employed for the colocalization study. As shown in Figure 3, we can draw the results that **pH-A** merges well with fluorescence images of lysosome dye (Pearson coefficient 0.87, Figure 3F) and that **pH-B** merges very well with fluorescence images of lysosome dye (Pearson coefficient 0.95, Figure 3L).

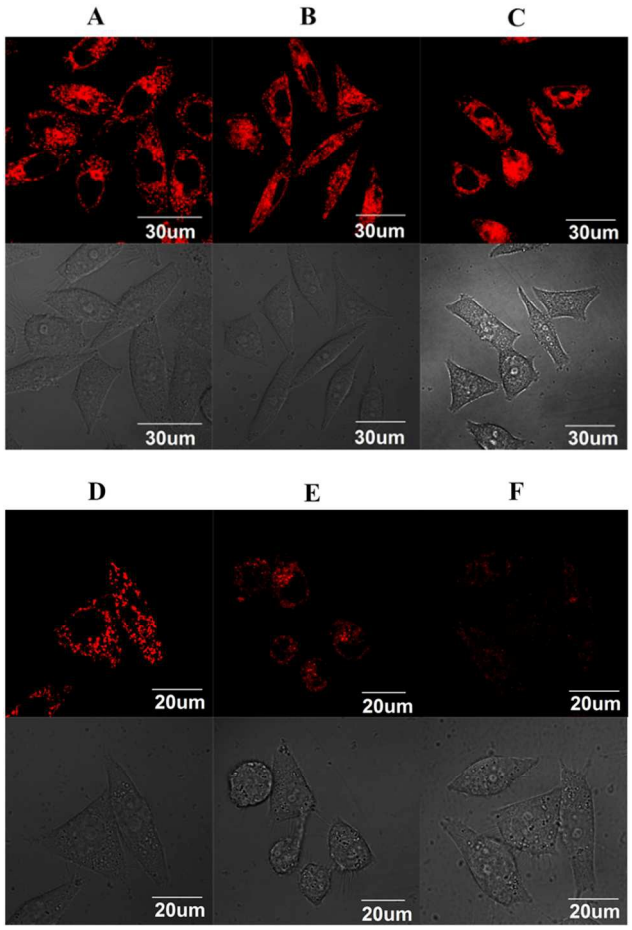


Figure 4. Fluorescence images of MCF-7 cells incubated with fluorescent probes **pH-A** and **pH-B**. A: Cells are incubated with 8μM **pH-A** for 15min. B: Cells are incubated with 8μM **pH-A** for 15min and then treated with 1.6mM chloroquine for 50min. C: Cells are incubated with 1.6 mM chloroquine for 30min and then treated with 8μM **pH-A** for 15min. D: Cells are incubated with 10μM **pH-B** for 20min. E: Cells are incubated with 10μM **pH-B** for 20min and then treated with 1.6mM chloroquine for 50min. F: Cells are incubated with 1.6 mM chloroquine for 30min and then treated with 10μM **pH-B** for 20min. λ_{ex}=635nm, λ_{em}=655-755nm

To evaluate the probes performance of pH, chloroquine is used for MCF-7 cells. Chloroquine is an alkaline drug which can concentrate lysosomes and raise pH of the lysosomes simultaneously.[23, 24] We divide the imaging experiments into three sets. First one is control experiment, in which cells are incubated with probes without adding chloroquine (Figure 4A and 4D). In second set, the cells stain with the probes and then are treated with chloroquine (Figure 4B and 4E). In the third set, chloroquine addition and treatment are in advance of the cells labelling with probes (Figure 4C and 4F).

For **pH-A**, the intracellular fluorescence signals are always strong, no matter adding chloroquine or not, and no matter the order of chloroquine treatment and **pH-A** staining. There is little difference of fluorescence intensity of **pH-A** between the cells in Figure 4A, 4B and 4C. As is known, lysosomes are acidic (pH 4-

6). Chloroquine can lift the lysosomal pH to some extent, but the final pH is still in acidic range. Hence, the fluorescence of **pH-A**, with higher pKa value (7.2) closed to neutral pH, is slightly affected by chloroquine. In contrast, for **pH-B**, there is very large difference of fluorescence intensity of between the cells in Figure 4D, 4E and 4F. This difference can be ascribed to the acidic pKa of **pH-B** at 6.1. Compared with Figure 4D (control), Figure 4E and 4F demonstrated remarkable lower fluorescence intensities. Moreover, the fluorescence in Figure 4F is too weak to be detectable, which is even weaker than Figure 4E. This phenomenon indicates that pre-treatment of chloroquine could reduce the amount of **pH-B** accumulated in lysosomes. Therefore, lysosomal pH increased by chloroquine not only quenches the fluorescence of **pH-B** but also lowers the cells uptake of **pH-B**. And based on the above results of the two pH probes, we can draw a conclusion that the probes can monitor changes of pH in lysosomes.

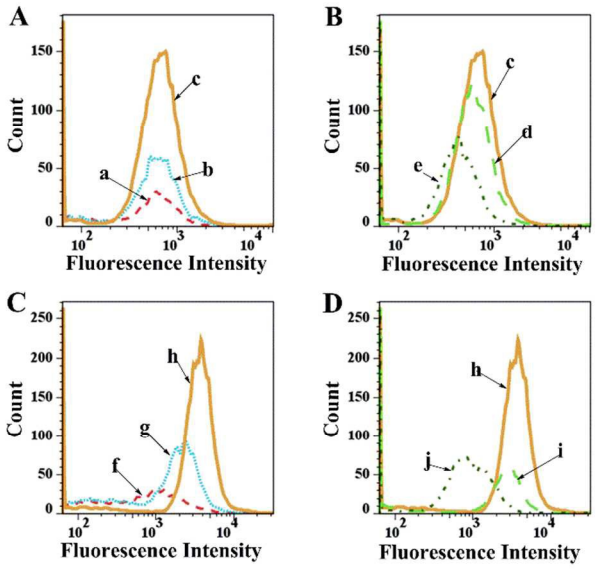


Figure 5. Flow cytometric analysis of MCF-7 cells. A: MCF-7 cells incubated with **pH-A** (6 μM) at pH=4.5 (a), 5.5 (b), 6.5 (c). B: MCF-7 cells incubated with **pH-A** (6 μM) at pH=6.5 (c), 7.5 (d), 8.6 (e). C: MCF-7 cells incubated with **pH-B** (8 μM) at pH= 4.5 (f), 5.5 (g), 6.5 (h). D: MCF-7 cells incubated with **pH-B** (8 μM) at pH= 6.5 (f), 7.5 (g), 8.6 (h).

In order to further prove applicability of the probes, flow cytometry (FCM) is used to quantitatively evaluate changes of fluorescence intensity with pH. Kim's group, Ohkuma's grounp and Baggaley'group report that the intracellular pH changes with extracellular pH.[11, 22,25] MCF-7 cells are incubated with the probes at pH=4.5, 5.5, 6.5, 7.5, 8.6 and then they are used for FCM. The changes of intracellular fluorescence intensity indicated by the shift of the fluorescence signal are measured in Figure 5. The number of the cells with fluorescence intensity increased as the cells are incubated with **pH-A** at pH from 4.5 to 6.5 (Figure 5A, a, b, c). The fluorescence intensity increases little because of its pKa close to neutral. And the fluorescence intensity decrease, as the cells are incubated with **pH-A** at pH from 6.5 to 8.6 (Figure 5B, c, d, e). We can know that **pH-A** is able to monitor change of pH in the cells. The similar results are obtained from flow cytometry experiment that cells incubated with **pH-B** are at pH=4.5, 5.5, 6.5, 7.5, and 8.6 (Figure 5C and 5D). The change magnitude of fluorescence intensity of **pH-B** is greater than that of **pH-A** via Figure 5A, 5B, 5C, and 5D, which also proves the above result that there is large difference of fluorescence intensity of **pH-B** after the cells incubated with chloroquine. And that also proves that the probes can monitor the changes of pH in the cells.

In summary, we have prepared two near-infrared fluorescent probes for pH (**pH-A**, **pH-B**). And the fluorescence quantum yields of **pH-A** and **pH-B** are 0.19, 0.095. As far as we know, it is not reported for pH in near-infrared range of fluorescent probes that there is a high fluorescence quantum yield (**pH-A,** Φ_f 0.19 in methanol and **pH-B,** Φ_f 0.095 in methanol) and its pKa close to neutral pH. And the applicability of these probes has been confirmed by monitoring the raise of lysosomal pH in MCF-cells stimulated by chloroquine. We believe that these pH probes may find more applications in detecting pH values in vivo and exploring the function of cells.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of the synthesis, NMR spectroscopic, ^{1}HNMR, ^{13}CNMR, cell culture and confocal imaging]. See DOI: 10.1039/b000000x/

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The near-infrared fluorescent probes for pH, named **pH-A** and **pH-B**, for labeling cells to produce the high resolution fluorescent images reflect changes of intracellular pH.