Rodamine-based lysosome-targeted fluorescence probes: high pH sensitivity and their imaging application in living cells†

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Two rhodamine-based pH probes (RhP and RhPA) were synthesized via click reaction. The probes exhibited high pH sensitivity and selectivity with significant fluorescence intensity enhancement. Cell imaging experiments demonstrated RhPA was a good lysosomes targeting probe in living cells with low cytotoxicity and excellent photostability.

In recent years, owing to the abnormal changes of cells may cause various disease, researchers are becoming more concerned about the health state and death of living cells. As far as we know, there are plenty of ways to defined cell death, take practical microscopy approach for instance,1 including: membrane integrity detection by DNA intercalating probes, protein expression by GFP (Green Fluorescent Protein), dielectric measurements and intracellular pH value measurements.2,3 Among others, intracellular pH value is widely used as an indicator for the general health of cells, and it really plays an important role in cells, such as enzyme and tissue activities, cell growth (including proliferation and apoptosis), endocytosis, multidrug resistance, calcium regulation (ion transport) and other cellular processes.4-9 The pH value of certain tumor tissues and organs (pH 6.0 - 6.5) possess lower compared to normal ones (pH 7.2-7.4).10,11 Moreover, at the cellular level, proton concentrations are not uniformly distributed: the cytoplasma is slightly alkaline (pH ~7.2), whereas the pH value of some intracellular organelles (lysosomes, endosomes and autophagosome) is in the range of 4.0 - 6.0.6,12-19 As the endpoint of the endocytic pathway, Lysosomes were found to be linked with the three major morphologically distinct pathways of cell death: apoptosis, type II programmed cell death and necrosis.20, 21 Hence, the study of sensing intracellular weakly acidic pH value, especially in lysosomes, has attracted increasing attention of researchers.

Recently, some fluorescent indicators have been developed to measure pH changes of environment or in living cells,5, 9 but only a few of them are suitable for pH detection in weakly acidic environments, and most of them exhibited poor membrane permeability and bad water solubility. Therefore, designing pH fluorescent probes with weakly acidic pKa and excellent membrane permeability are still desirable. Considering that fluorescent dyes could be used for visual imaging, we intend to choose a suitable dye and prepare some lysosome-targeted pH probes. According to previous studies of other researchers, rhodamine dyes not only have excellent photostability, photophysical properties and suitable water-solubility, but also have the potential for targeting lysosomes without introducing any other lysosome-located groups.15, 22-26 Since the cyclisation equilibrium in rhodamine derivates were widely used,27, 28 we believe that probes based on rhodamine dyes are still of high interest, particularly for pH sensing.10, 29-31

Scheme 1

Scheme 2

Herein, two rhodamine B based probes were synthesized via click reaction (Scheme 1). 1,2,3-triazole was introduced as an ideal bridge to improve the biocompatibility as well as water-solubility,32 moreover to avoid “alkalizing effect” we adopted benzene rather than any other aliphatic amines 33. The pH sensitive spirolactam structure of probes (RhP and RhPA) remains closed and non-fluorescent in the neutral environment; whereas weakly acidic condition leads to the ring-opening of spirolactam and the probes exhibit strong emission spectra (Scheme 2). In addition, the difference between RhP and RhPA is that RhPA comprises one more amide bond, which might serve as additional protonate groups under acid conditions. The structures of probes and intermediates were confirmed by 1H

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NMR, $^{13}$C NMR and ESI-MS.

With the targeted probes in hand, we firstly investigated the fluorescent response of RhP and RhPA (5 μM) to pH at room temperature, and Britton-Robinson buffer solution is used for turning pH values. As shown in Fig. 1, when exciting RhP and RhPA at 540 nm, non-fluorescent were found under neutral condition, while with the pH decreasing, the fluorescent intensity of the two probes increased significantly at 583 nm. RhP increased about 35 fold from pH 7.6 to 4.0 (Fig. 1A), and the quantum yield increased from 0.01 to 0.16; while RhPA increased about 75 fold from pH 8.0 to 4.4 (Fig. 1B), and the quantum yield increased from 0.03 to 0.45. The results were definite attribute to the transformation of the ring-opened tautomer of the rhodamine fluorophore. According to the pH titration, the highly sensitive pH range of RhP and RhPA could be found to be from 4.0 to 6.0 and from 4.4 to 6.5, respectively.

Moreover, the pK$_a$ values of the two probes were calculated as 4.79 for RhP and 5.23 for RhPA (via the Henderson-Hasselbach-type mass action equation, as shown in Figure S1 and Figure S2). The weak acidic pK$_a$ was exactly suitable for the application of imaging of lysosomes (intracellular acidic organelles) in living cells.

Fig. 1 A) Fluorescence spectral changes of RhP (5 μM) in B-R buffer solution at different pH values ($\lambda_{ex} = 540$ nm), and maximum emission intensity was measured at 583 nm. Inset: Plot of the emission fluorescence intensity at 583 nm. pH 3.60, 4.00, 4.19, 4.40, 4.62, 4.80, 5.01, 5.21, 5.41, 5.59, 5.82, 6.00, 6.21, 6.42, 7.01, 7.61. B) Fluorescence spectral changes of RhPA (5 μM) in B-R buffer solution at different pH values ($\lambda_{ex} = 540$ nm), and maximum emission intensity was measured at 583 nm. Inset: Plot of the emission fluorescence intensity at 583 nm. pH 3.60, 4.00, 4.19, 4.40, 4.62, 4.80, 5.01, 5.21, 5.41, 5.59, 5.82, 6.00, 6.21, 6.42, 7.01, 7.61, 8.05.

Fig. 2 A) Pink bars: Fluorescence response of RhP (5 μM) at 583 nm toward other competitive ions in B-R buffer solution (pH 4.20). Grey bars: selectivity of RhP (5 μM) for pH at 583 nm toward other selected interferences in B-R buffer solution (pH 7.50). B) Pink bars: Fluorescence response of RhPA (5 μM) at 583 nm toward other competitive ions in B-R buffer solution (pH 4.20). Grey bars: selectivity of RhPA (5 μM) for pH at 583 nm toward other selected interferences in B-R buffer solution (pH 7.50). Na+: 150 mM; K+: 75 mM; Mg$_2^+$, Ca$_2^+$, Zn$_2^+$: 5 mM; other ions: 0.1 mM.

Subsequently, to further verify the selectivity and anti-interference of our probes, we examined the fluorescent response of RhP and RhPA for H$^+$ in the presence of other cations at different pH. As shown in Fig. 2, at pH = 7.50, with the addition of Na$^+$ (150 mM), K$^+$ (75 mM), Li$^+$, which are plentiful in living cells; heavy and transition-metal cations such as Hg$_2^{2+}$, Pb$_2^{2+}$, Mn$_2^+$, Co$_2^+$, Ni$_2^+$, Ag$^+$, Cu$_2^{2+}$, Cd$^{2+}$, no fluorescence enhancement were found for RhP and RhPA. Mg$_2^+$, Ca$_2^+$, Zn$_2^+$ led to feeble fluorescence enhancement of RhPA, however the concentration of Mg$_2^+$, Ca$_2^+$, Zn$_2^+$ in vivo was much lower than 5 mM, thus the effect of these ions could be neglected. Meanwhile, the influence of these metal cations was also studied at pH = 4.20, and the fluorescence intensity of RhP and RhPA in the presence of these cations were almost the same as the intensity at pH = 4.20. These results demonstrated that RhP and RhPA had high specific fluorescent response to acidic pH without any effects of the complicated intracellular condition and were suitable for imaging of living cells. Additionally, the reversibility of the sensor was also of great importance to the practical application, so we detected the reversible transformation.
of our probes by pH titration. As shown in Fig. 3, an apparent
decline of the fluorescence intensity of the two probes were found
with the addition of OH⁻ (NaOH), when H⁺ (HCl) was added to
the solution and the pH value transferred to the original value
again, the fluorescence intensity of the two probes was also
recovered. This circulation could be repeated for at least ten times,
and the reversible pH response would also be benefit for
fluorescent intracellular pH imaging.

Finally, RhP and RhPA were applied to imaging and sensing
of the pH in living cells (Hela cells) by confocal laser scanning
microscopy analysis. To determine the distribution of the probes
in living cells, Hela cells were co-stained with commercially
available nucleus-specific, lysosome-specific and mitochondrion-
specific staining probes, NucBlue® Live Cell Stain (one drop per
milliliter), LysoTracker Green DND-26 (1 μM) and MitoTracker
Green FM (1 μM). As shown in Fig. 4, the bright red emission
which mainly distributed in cytoplasm indicating that RhP and
RhPA could get into cells (Fig. 4 c, g, k and o). More
interestingly, we noticed that the subcellular regions stained with
RhP not only matched those stained with LysoTracker Green
very well, but also matched well with MitoTracker Green staining
(Fig. 4 d and h), and the subcellular regions stained with RhPA
only matched well with LysoTracker Green staining (especially
contrast l with p of Fig. 4). These preliminary results proved that
RhP and RhPA had different locations in living cells.

Furthermore, in order to substantiate whether only RhPA
really could be used for lysosome (acidic compartment) specific
staining, a qualitative co-localization index was measured by
choosing a Region of Interest (ROI) in one cell. As Fig. 5
illustrated, the green line means the signal of LysoTracker Green
and MitoTracker Green from the region we chose and the red line
represents the signal of RhP or RhPA from the same region. The
two coordinate graphs A) and B) of RhP in Fig. 5 elucidate that
no matter the green line or the red line the peaks were all in the

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Fig. 3 A) pH reversibility research of RhP (5 μM) between pH ~4.30 and ~10.40. B) pH reversibility research of RhPA (5 μM) between pH ~4.20 and ~10.40.

Fig. 4 Confocal microscopy images of the intracellular distribution of lysosomes (incubation 30 min). a – d: Hela cells was stained with one drop NucBlue (blue channel), 1 μM LysoTracker Green (green channel) and 5 μM RhP (red channel), e – h: Hela cells was stained with one drop NucBlue (blue channel), 1 μM MitoTracker Green (green channel) and 5 μM RhP (red channel), i – l: Hela cells was stained with one drop NucBlue (blue channel), 1 μM LysoTracker Green (green channel) and 5 μM RhPA (red channel), m – p: Hela cells was stained with one drop NucBlue (blue channel), 1 μM MitoTracker Green (green channel) and 5 μM RhPA (red channel).

Fig. 5 a – d: confocal microscopy images of Hela cells co-stained with RhP (5 μM), LysoTracker Green (1 μM) and NucBlue (one drop). e – i: confocal microscopy images of Hela cells co-stained with RhP (5 μM), MitoTracker Green (1 μM) and NucBlue (one drop). i – l: confocal microscopy images of Hela cells co-stained with RhPA (5 μM), LysoTracker Green (1 μM) and NucBlue (one drop). m – p: confocal microscopy images of Hela cells co-stained with RhPA (5 μM), MitoTracker Green (1 μM) and NucBlue (one drop). A) – D): Intensity profile of regions of interest (ROI) across Hela cells (green line – green channel, red line – red channel)

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same position, suggesting that this probe not only located in lysosomes, but also existed in mitochondria. The other two coordinate graphs C) and D) of in Fig. 5 illustrated that RhPA only co-stained with LysoTracker Green (the peaks of the green line and the red line were in the same position). However, although the signal from cells which co-stained with MitoTracker Green were in the same position, it is obvious that RhPA had strong signal intensity while MitoTracker Green exhibited weak signal intensity.

**Fig. 6** Cytotoxicity of LysoTracker Green, MitoTracker Green, RhP and RhPA on HeLa cells. The concentration of LysoTracker Green and MitoTracker Green: 0 μM, 0.156 μM, 0.312 μM, 0.625 μM, 1.250 μM, 2.500 μM, 5.000 μM (from left to right). The concentration of RhP and RhPA: 0 μM, 0.625 μM, 1.250 μM, 2.500 μM, 5.000 μM, 10.000 μM, 20.000 μM, 40.000 μM (from left to right).

Besides intracellular pH imaging, the cytotoxicities of RhP, RhPA and the two commercial dyes (LysoTracker Green and MitoTracker Green) were also detected. As illustrated in Fig. 6, no obvious toxicities were found for RhP in cells even at the concentration of 40 μM, and no obvious toxicities were found for RhPA at the concentration of 10 μM. However, the two commercial dyes were more toxic. These results suggested that compared with the two commercial dyes, our probes were much less toxic, it also suggested that our probes were more beneficial for biological applications.

**Conclusions**

In summary, two rhodamine-based pH-sensitive probes have prepared via click reaction. They both have excellent selectivity and sensitivity in aqueous solution, moreover metal cations have no significant interference on pH sensing and the two probes were fully reversible mainly within the pH range from 4.2 to 10.4. RhP and RhPA exhibited low cytotoxicities and excellent photostability. The confocal co-localization imaging experiment of the two probes indicated that RhP and RhPA had good biocompatibility, and suitable for detecting the acidic region of living cells. Especially, RhPA could be successfully used for lysosomes targeting.

This work was financially supported by the National Program on Key Basic Research Project of China (973 Program, 2012CB720603 and 2013CB328900), the National Science Foundation of China (Nos. 21232005, 21321061, J1310008 and J1103315), and the Specialized Research Fund for the Doctoral Program of Higher Education in China (2012011130006). We also thank the Analytical & Testing Center of Sichuan University for NMR analysis.

**Notes and references**

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†Electronic Supplementary Information (ESI) available: [spectra of target probes and intermediates, detail experimental procedures]. See DOI: 10.1039/b000000x/

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