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Imaging of lysosomal pH changes with a fluorescent sensor containing a novel lysosome-locating group†

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Rlyso, a highly selective and sensitive pH sensor, can stain lysosomes with a novel lysosome-locating group, methylcarbitol. Rlyso was successfully used to detect lysosomal pH changes during apoptosis or induced by chloroquine while avoiding the "alkalizing effect" on lysosomes of current lysosomal probes with nitrogen-containing sidechains.

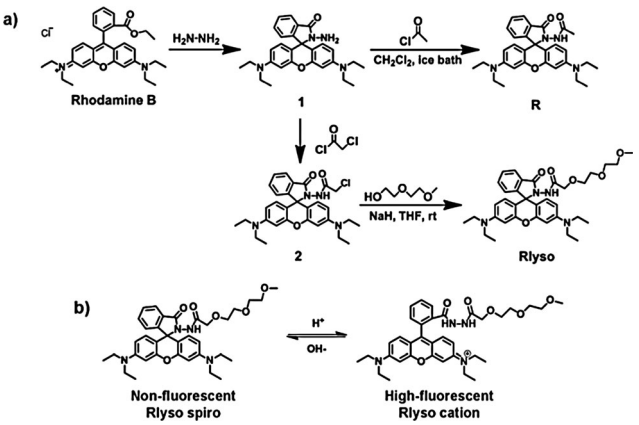
Eukaryotic cells contain compartments with different degrees of acidity. For example, the cytoplasma is slightly alkaline at about pH 7.2, whereas organelles, such as lysosomes and endosomes, have intracompartmental pHs of 4.0–6.0.[1] Lysosomes contain approximately 50 different degradative enzymes that are active at acidic pH (~5).[2] Abnormal lysosomal or endosomal acidification is associated with various pathological conditions. In particular, the lysosomal proton gradient is lost at an early time point in the apoptosis process.[3]

Chemosensors have found widespread application in the fluorescence imaging of various analytes in live cells,[4] tissues[5] and organisms[6] because of their rapid, nondestructive, selective and sensitive advantages of emission signals. The current specific lysosomal pH sensors, including the commercial LysoSensor probes, are weak bases[7] that selectively concentrate in acidic organelles upon protonation. This protonation also relieves the fluorescence quenching of these dyes, which results from the photoinduced electron transfer (PET) by their amine-containing side chains such as 4-(2-hydroxyethyl)morpholine and *N*,*N*-dimethylethylenediamine. Unfortunately, these lysosomal probes can exhibit an "alkalizing effect" on lysosomes, such that longer incubation with these probes can induce an increase in lysosomal pH.[7f] Moreover, due to the inadequate fluorescence quenching of the PET mechanism, such probes often exhibit high, nonspecific background fluorescence signals inside cells. Therefore, the development of alternative recognition mechanisms for H^+ and lysosome-locating groups that do not have nitrogen atoms would be of great significance and practical value.

Rhodamine dyes have been extensively used as fluorophores by virtue of their excellent photophysical properties. More importantly, a spirolactam ring exists in the "ring-closed" state without H^+ and the rhodamine dyes are completely nonfluorescent, thus effectively preventing the interference of background fluorescence. Addition of H^+ leads to ring opening, which results in significant fluorescence enhancement. Based on this mechanism, some fluorimetric pH sensors have been exploited.[7a,8]

Here, we report a rhodamine-based pH fluorescent sensor **Rlyso**, in which a novel lysosome-locating group, methylcarbitol, is first introduced. A contrasting molecule, **R**, which is free of the methylcarbitol group, was proven to not partition into acid organelles. The respective log *P* values of the spiro and the open-ring cationic species of **R** and **Rlyso** were calculated to further verify the lysosome-locating character of **Rlyso**.

R and **Rlyso** were readily synthesised from rhodamine B *via* a two- and three-step procedure, respectively (Scheme 1a). The fluorescence spectra of **Rlyso** at various pH are recorded in Fig. 1a. **Rlyso** was almost nonfluorescent at pH 7.4 because of its stable "ring-closed" form. The fluorescence was significantly enhanced as the H^+ concentration was increased. There is more than a 50-fold increase in the emission intensities within the pH range of 7.4–4.5, demonstrating that **Rlyso** is a sensitive acid-responsive sensor, the large fluorescence enhancement being due to the H^+-induced spirolactam ring opening. In Fig. 1b, the changes in the fluorescence intensity as a function of



Scheme 1 (a) Synthesis of **R** and **Rlyso**. (b) Proton triggered ring opening of **Rlyso**.

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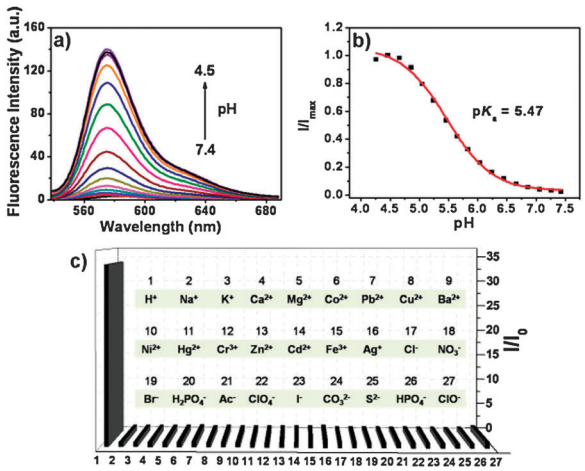

Fig. 1 (a) Fluorescence spectra of **Rlyso** at various pH in Britton–Robinson buffer solution, λ_{ex} = 525 nm. (b) pH titration curve of **Rlyso**, λ_{ex} = 559 nm, λ_{em} = 578 nm. (c) Fluorescence responses (578 nm) of **Rlyso** to miscellaneous ions in water: 50 μM for H^+, 200 μM for the other ions.

pH yielded a pK_a of 5.47, indicating that **Rlyso** can detect minute pH changes in the 4.5–6.0 range, which covers both normal and abnormal lysosome pH. **Rlyso** can respond to H^+ and fluorescence intensity saturation is reached within several seconds at pH 4.88 and 5.58, respectively (Fig. S2, ESI†), which is vital to detect lysosomal pH changes in real time. In a similar manner, the pH titration curve of **R** yielded a pK_a of 5.59 (Fig. S3, ESI†), and maximum fluorescence was achieved at pH 4.8.

The selectivity of **Rlyso** for H^+ was determined *via* fluorescence spectroscopy (Fig. 1c). **Rlyso** did not exhibit observable fluorescence enhancement in the presence of common cations such as Na^+, K^+, Ca^{2+} and Mg^{2+}, which are ubiquitous in mammalian cells, as well as heavy and transition-metal ions such as Co^{2+}, Pb^{2+}, Ni^{2+}, Hg^{2+}, Cr^{3+}, Zn^{2+}, Cd^{2+}, Fe^{3+}, Ag^+ and Cu^{2+}. Moreover, various common anions (Cl^-, NO_3^-, Br^-, $H_2PO_4^-$, Ac^-, ClO_4^-, I^-, CO_3^{2-}, S^{2-}, and HPO_4^{2-}) exhibited negligible interference on **Rlyso** activity. **Rlyso** was unreactive to OCl^-, despite the presence of a hydrazo group similar to dibenzoylhydrazine, which can be selectively oxidised into dibenzoyl diimide by OCl^- anion.[9] The competition experiments reveal that the H^+-induced fluorescence response was unaffected in the presence of background ions (Fig. S4, ESI†). Fig. S5 (ESI†) shows the fluorescence intensity of **Rlyso** in a buffer solution when the pH is switched between 4.8 and 7.4 five times. Approximately 93.2% of the original signal was successfully generated. The high selectivity of **Rlyso** over interfering species along with the reusability of protons indicate that **Rlyso** has significant potential as a pH sensor for practical applications.

Lysosomes were shown to have proton-pumping vacuolar ATPases, which maintain the lumenal environment at a pH range of 4.6–5.0.[10] The acidity of the lysosome is vital to the optimal activities of various hydrolases.[2] Confocal fluorescence microscopy results show that **Rlyso** exhibited observable red punctate fluorescence near the perinuclear regions of live cell lines (MCF-7, Hela and Raw 264.7), whereas negligible fluorescence signals were observed in the cells cultured with **R** (Fig. S6, ESI†). These results demonstrate that **Rlyso** stains the acidic vesicles but **R** does not. To further determine the subcellular distribution of **Rlyso**, a commercially available

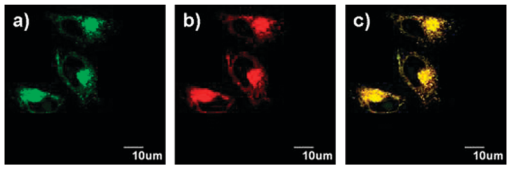

Fig. 2 **Rlyso** co-localizes to lysosomes in live MCF-7. Cells were stained with: (a) 1 μM LysoSensor Green, (b) 10 μM **Rlyso**. (c) Areas of co-localization appear in yellow. **Rlyso** and LysoSensor Green were excited at 559 nm and 488 nm, respectively. The fluorescence images were recorded at 575–620 nm (**Rlyso**) and 495–515 nm (LysoSensor Green), respectively.

lysosome-specific staining probe, LysoSensor™ Green DND-189, was used to co-stain cells with **Rlyso**. Colocalization was quantified using Pearson's sample correlation factors (R_r). Fig. 2 and Fig. S7 (ESI†) show that the distribution of **Rlyso** is nearly the same as that of LysoSensor Green in all three cell lines. The co-localization of **Rlyso** with LysoSensor Green was observed in 98% of Hela cells, 98% of MCF-7 cells and 95% of Raw 264.7 cells, suggesting that **Rlyso** selectively stains lysosomes in live cells.

The structure–activity relationship approach expresses physicochemical properties of probes numerically, and relates these values to the staining or non-staining of lysosomes.[12] Hydrophilicity–lipophilicity is modelled by the logarithm of the water/octanol partition coefficient (log *P*). Probes specifying lysosome accumulation are assigned numerically by the following criteria: $+5 > \log P_{\text{neutral species}} > 0$; $-5 < \log P_{\text{cation}} < 0$.[12,13] Values of log *P* values of the spiro and the open ring cationic species of **R** and **Rlyso** are listed in Table 1. The value of $\log P_{\text{neutral species}}$ of **Rlyso** is in the range 0 to +5 suggesting its easy membrane permeability. In the case of **Rlyso** cationic open ring species, $\log P_{\text{cation}}$ falls in the −5 to 0 range, which is hydrophilic and not very membrane permeant. So even if **Rlyso** is generated in the low pH lysosomal environment, it would probably become trapped within the lysosome. However, **R** spiro species is very lipophilic and it is expected to be substantially taken up into the various cell membranes. Consequently membrane permeability will be poor. Furthermore, the **R** cationic open ring species is still lipophilic. Being in the range to permit ready membrane permeability it can diffuse out of the lysosome into the cytosol. Within this less acid environment it will presumably revert to the non-fluorescent spiro species.

Weakly basic substances can cause the leakage of protons out of lysosomes in the form of protonated bases. The concentration of chloroquine required to cause an increase in lysosomal pH is considerably lower than that of other weak bases.[14] In this study, **Rlyso** was used to detect the chloroquine-induced lysosomal pH changes in macrophages. The normal pH inside macrophage lysosomes seems to be 4.7–4.8,[15] and **Rlyso** exhibited strong fluorescence in these cells

Table 1 Values of log *P* of **R** and **Rlyso**

Dye species	log P^a
R spiro	7.3
Rlyso spiro	4.9
R cationic open ring	1.6
Rlyso cationic open ring	−0.8

[a] Calculated with the Hansch and Leo manual procedures.[11]

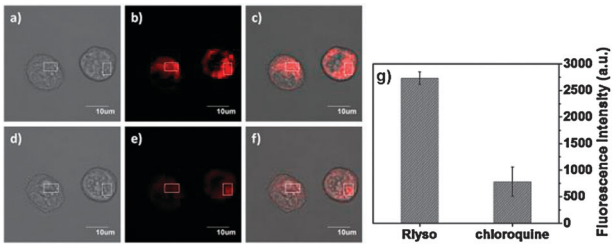

Fig. 3 Fluorescence images of **Rlyso** in mouse macrophages stimulated with chloroquine. (a–c) Images of the stained cells before chloroquine-stimulation; (d–f) images of cells in (a–c) exposed to 100 μM chloroquine for 2 min. (g) Relative fluorescence intensity values. The probe was excited at 559 nm and the fluorescence was collected at 575–620 nm.

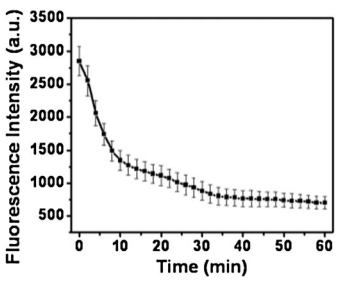

Fig. 4 Time dependent relative fluorescence intensity of **Rlyso** in Hela cells undergoing apoptotic death induced by dexamethasone (2 μM).

before treatment with chloroquine (Fig. 3b). Addition of 100 μM chloroquine clearly reduced the fluorescence intensities (Fig. 3e). To determine the intensity of each image, two areas in the image were chosen to represent the lysosomal regions of the cell. The values obtained for each case were subsequently averaged (Fig. 3g). Referring to the titration curve shown in Fig. 1b, the lysosomal pH increased by 1.20 ± 0.04, which is nearly consistent with the literature.[14] This result indicates that **Rlyso** is a lysosomal pH-responsive sensor in cell biology.

Photobleaching is a common problem for many organic dyes, with the reporting dyes often compromising the temporal monitoring of dynamic events inside cells.[7a] Fig. S8 and S9 (ESI†) indicate the high photostability of **Rlyso** under environmental and biological conditions.

It is known that the cytosol is acidified during apoptosis,[3,7b] which might be due to lysosomal proton release.[3] To verify the lysosomal pH change during apoptosis, dexamethasone was used to induce apoptosis. **Rlyso** was then used as the pH sensor. Fig. 4 shows that the addition of dexamethasone caused time-dependent decreases in the fluorescence intensity, which indicates increases in lysosomal pH in accordance with the results in Fig. 1b. Furthermore, after the dexamethasone treatment, the fluorescence signal of **Rlyso** significantly weakened within 10 min and observable apoptosis-related morphological changes were achieved 30 min later (Fig. S10, ESI†). This observation is consistent with those of a previous study, which reported that the loss of the lysosomal proton gradient and the occurrence of a leakage of lysosomal proteases are events that occur early in apoptosis.[16]

MTT assays revealed that the cell viabilities of HeLa cells and Raw 264.7 cells were not affected by incubation with 10 μM **Rlyso** and **R** for 12 h (Fig. S11, ESI†).

In summary, a fluorogenic rhodamine-lactam pH sensor, **Rlyso**, exhibited high selectivity and sensitivity for intracellular pH, as well as low cytotoxicity and high photostability. It can selectively stain lysosomes in live cells. This staining ability is due to the presence of the methylcarbitol group, which avoids the "alkalizing effect" on lysosomes by the current lysosomal sensors with nitrogen-containing sidechains. Importantly, **Rlyso** was used to quantitatively detect the chloroquine-induced increase in lysosomal pH and monitor changes in the acidity of lysosomes during apoptosis in live cells.

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