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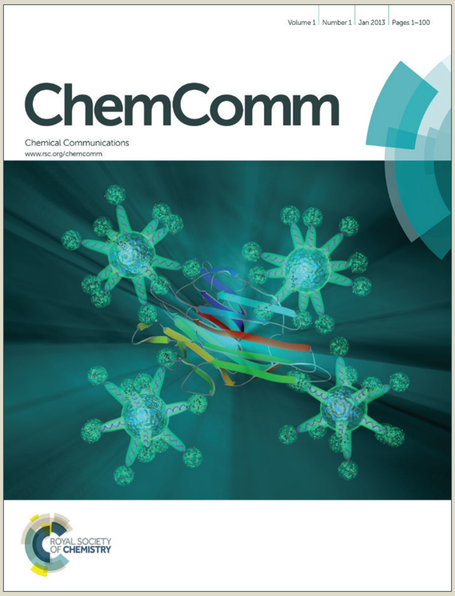

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ARTICLE TYPE

A Switchable Peptide Sensor for Real-time Lysosomal Tracking†

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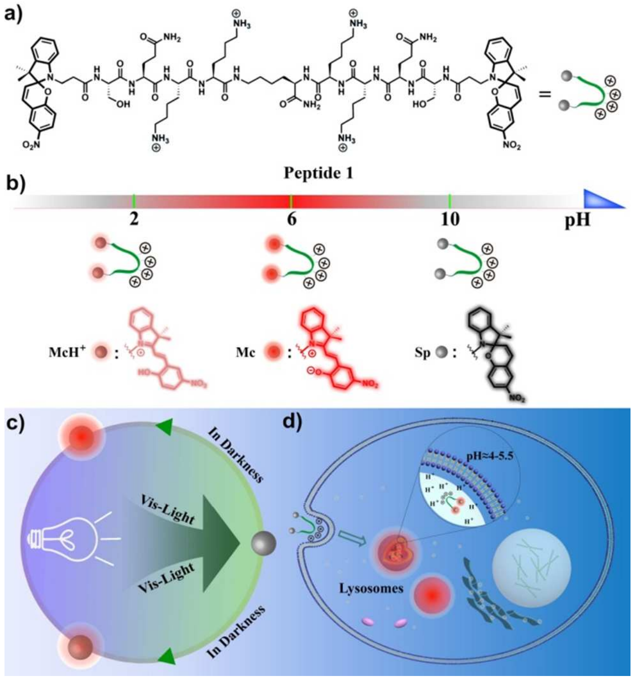
A bis-spiropyran functionalized peptide 1, which exihibits good cell-permeability, excellent biocompatibility and low cytotoxicity, has been developed for reversible and real-time lysosomal tracking.

Lysosomes, as membrane-bound organelles, contain a large variety of hydrolytic enzymes and secretory proteins that are active at an acidic luminal pH range (~4.0-5.5).[1] Lysosomes are not only serving as the terminal degradative compartment of live cells, but also involved in many physiological processes including metabolism, intracellular transport, and cell membrane recycling as well as apoptosis.[2] However, lysosomal malfunctions can lead to different pathophysiological conditions,[3] especially to cancer-related diseases.[4] Thus, effective strategies to visualize lysosomes of cancer cells are of significant interest to prevent or treat tumor invasion and metastasis and will pave the way for lysosome-related disease diagnosis and therapy.

Very recently, large efforts have been devoted to the development of fluorescent sensors employed to study lysosomal functions in live cells, which includes polymers,[5] nanoparticles,[6] modified quantum dots,[7] and macromolecules, such as dextran labeled with a fluorophore.[8] Although these sensors can be exploited to track the intracellular lysosomes efficiently, they also exhibit the following limitations: (i) it is difficult to target them to specific lysosomal lumen with high spatial and temporal resolution; (ii) they often suffer from poor solubility in water and display poor biocompatibility; and (iii) they show irreversible switching cycles limiting the span of time-lapse imaging. Considering these difficulties, it is imperative to develop a new sensor to track lysosomes with good solubility, high efficiency and excellent biocompatibility. In addition, sensors capable of reversibly monitoring lysosomal imaging are also desirable. This ideal probe, however, has rarely been reported in the field of lysosomes.

Up to now, spiropyran derivatives were widely applied from data storage[9] to optical switching,[10] chemical sensing,[11] molecular machines[12] and logic gate,[13] based on the reversible structural isomerization from a colorless spiro-form (spiropyran, **SP**) to a colored open-form (merocyanine, **Mc**) by UV/Vis light illumination. However, pH-active spiropyran based sensor used to track intracellular lysosomes has been rarely explored. Moreover, it should be noted that the intrinsic photoswitchable property of spiropyran could be achieved by either pH or Vis-light realizing reversible lysosomal tracking. Therefore, we expected to employ a spiropyran fluorophore with a structurally simpler lysine-rich cationic peptide to be used for lysosomal tracking. This photoswitch would allow us to track and recognize lysosomes with high signal-to-noise upon spectroscopic changes imposed by pH-changes in aqueous solution.



Scheme 1 (a) Molecular structure of peptide **1**; (b) The coordinate represents the states of sensor at different pH values, purple sphere: protonated merocyanine (**McH$^+$**), red sphere: normal merocyanine (**Mc**), and gray sphere: spiropyran at a ring-closed state (**Sp**). (c) The photo-physical process of **1** at the corresponding pH; (d) The cartoon image shows the work principle of peptide **1** for live cell lines.

In this work, a bis-spiropyran functionalized peptide **1** (Scheme 1a) was specifically designed in anticipation of the tracking of lysosome upon the protonation of spiropyran moieties as well as the electrostatic interaction of positive charged lysine residues with negative charged lysomal membrane. This peptide has been proved to serve as a pH and light dual-reponsive photoswitch, in which the pH-induced ring open process was thoroughly studied. Moreover, **1** was well employed to reversible lysosomal tarcking within live cells. Peptide **1** was synthesized using a microwave-assisted solid-phase peptide method that commonly used for peptide-based biosensors,[14] which was isolated by preparative HPLC on a reversed-phase C-18 column in 99 % purity and 25 % yield, respectively (ESI†).

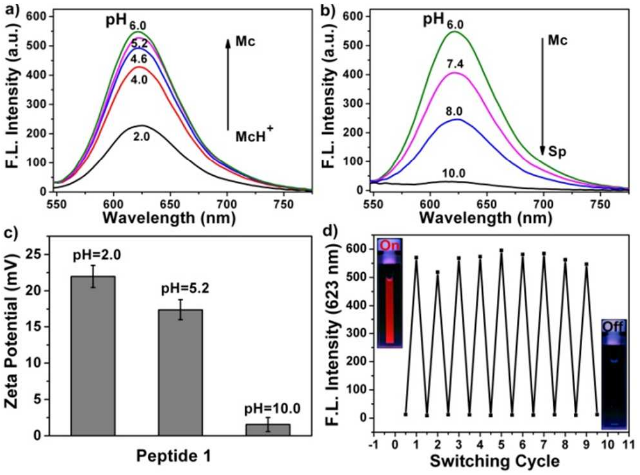


Fig. 1 (a) and (b) Fluorescence spectra of peptide **1** (50 μM) measured at different pH values in TBS (0.05 M Tris, 0.05 M NaCl, 20 °C) for 12 h (arrows show direction of intensity increase from 2.0 to 6.0 and intensity decrease from 6.0 to 10.0, respectively); (c) zeta potentials of peptide **1** measured at different pHs (pH=2.0, 5.2 and 10.0, respectively), results were presented as mean ± SD (n=3). (d) Sequential fluorescence switching cycles of peptide **1** controlled by Vis-light illumination for *ca.* 10 min and placed in darkness for 12 h in TBS (pH 5.2, 0.05 M Tris, 0.05 M NaCl, 20 °C), Inset: fluorescence images (UV light, 365 nm).

To identify the best pH-responsive conditions, spectroscopic studies of **1** were examined by UV/Vis absorption and fluorescence spectroscopy in Tris-HCl buffer solutions (TBS) at a pH range from 2.0 to 10.0. Below pH 2, **1** shows a sharp absorption band at 410 nm and relatively weak fluorescence emission at 623 nm (Fig. S1a-b, ESI†). The absorption band at 410 nm is attributed to the protonation of the phenolate anion of the merocyanine units (**McH**$^+$), which is consistent with a previous research.[15] In contrast, the fluorescence spectral changes of **1** at various pH values (4-6) are shown in Fig. 1a. Increasing the pH value gives rise to higher fluorescence intensity at 623 nm (Fig. S2b-S5b, ESI†), while the absorption band of **1** exhibits a bathochromic shift from 410 nm to 514 nm concomitantly (Fig. S2a-S5a, ESI†), a representative absorption of **Mc**.[15] As seen in Fig. 1a, the relative fluorescence intensities increased by approximately 3-fold from pH 2 to 6. Indeed, it is noteworthy that **1** exhibits strong fluorescence intensities increase in the pH range of *ca.* 4-6, which covers both normal and abnormal lysosome pH making **1** a promising fluorescent indicator (Scheme 1b) for lysosome. However, at pH ≥ 10.0, neither UV-Vis absorption nor fluorescence emission could be observed (Fig. 1b, and Fig. S8a-b, ESI†), suggesting the presence of the ring-closed state (**SP**). Besides, zeta potential measurements at specific pH values (pH 2.0, 5.2 and 10.0) indicated a decrease of surface-charge of **1** with the increase of pH values (zeta potential: 21.97 ± 1.52 mV, 17.38 ± 1.41 mV and 1.55 ± 0.97 mV at pHs 2.0, 5.2 and 10.0, respectively, Fig. 1c), which further confirmed that **1** exhibited three different state (**McH**$^+$, **Mc** and **Sp**) at pH range from 2 to 10. Moreover, we were excited that, as evident in Fig. 1d (pH 5.2), the photo switching of the fluorescence could be repeated many times without any apparent "fatigue" effects. The reversible switching processes could also be cycled under the pH range of pH 4-6 (details are not given). In contrast, the ring opening processes were also measured by UV light irradiation (Fig. S1c-S8c, S1d-S8d, ESI†). Interestingly, upon irradiation with UV light (365 nm), little or no changes of absorption and fluorescence were detected in contrast to the behaviour in darkness. This confirms that **1** effectively realizes the ring-opening process in aqueous solution with the concomitant occurrence of red luminescence by pH-changes rather than UV light irradiation. Moreover, with the irradiation of Vis-light, the ring-closing process of **1** occurs (Scheme 1c). To determine the physical parameters of **1** at various pH values from 2.0 to 10.0, the fluorescence quantum yield (Φ) was measured providing a maximum of 3.1 % with a lifetime for a single-exponential decay of 0.38 ns at pH 4-6 (Table S2, ESI†).

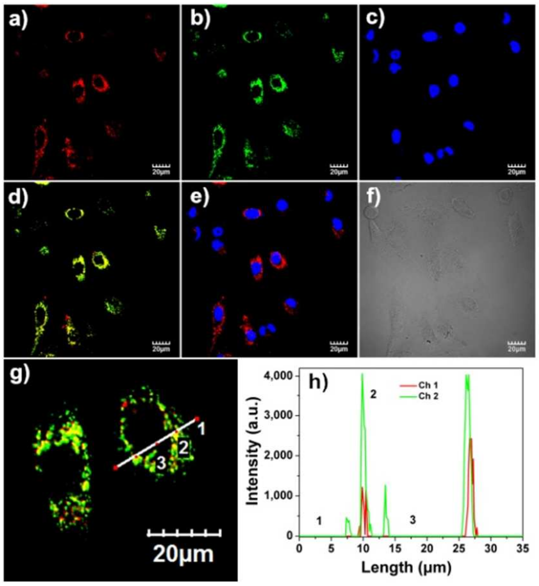


Fig. 2 Colocalization experiments using peptide **1**, Lyso Tracker Green DND-26 (LTG) and Hoechst 33258 in A549 cells. Cells were stained with (a) 10 μM peptide **1** (Channel 1: excitation: 515 nm, emission collected: 600-650 nm), (b) 0.1 μM LTG (Channel 2: excitation: 488 nm, emission collected: 500-550 nm) and (c) 10 μg/mL Hoechst 33258 (Channel 3: excitation: 405 nm, emission collected: 420-470 nm); (d) overlay of (a) and (b); (e) overlay of (a) and (c); (f) overlay of (a) and bright-field; (g) Partial enlarged image of (d) (cross-sectional analysis along the white line in regions of 1, 2 and 3); (h) Intensity profile of regions of interest across one A549 cell.

On the basis of the above results, we evaluated whether **1** can be efficiently taken up by cancer cells. After incubating live A549 cells with **1** for 1 h, an intense red luminescence was detected in the cytoplasm region, while no luminescence in the nucleus was observed (Scheme 1d, Fig. 2 and Fig. S9, S10, ESI†). We could deduce that the red signals most likely came from the acidic organelles (the intracellular lysosomes). To confirm this conclusion, a colocalization experiment of **1** with LysoTracker Green DND-26 (LTG) was performed. As shown in Fig. 2, cell images confirmed that the red and green signals from **1** (Fig. 2a) and LTG (Fig. 2b) originated from approximately the same cell region. The overlay images revealed that **1** mainly accumulates in the acidic lysosomes instead of the nucleus (Fig. 2d-e). In addition, amplified imaging of A549 cells shown in Fig. 2g and cross-sectional analysis (quantification of the luminescence intensity profile of **1** and LTG along the white line in image 2g, Fig. 2h) indicated that the luminescence indeed mainly stems from the lysosomes (spot 2) rather than the nucleus (spot 3). These facts suggest that **1** show good specificity toward lysosome and could act as a potential candidate of lysosomal tracking agent.

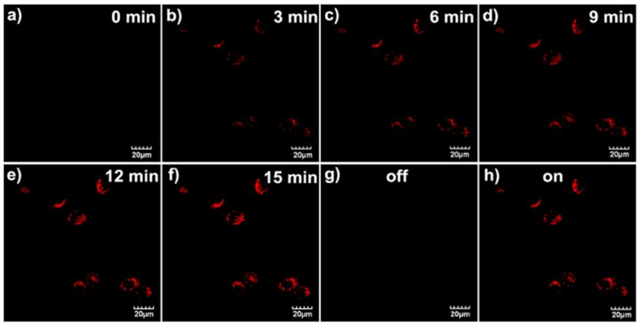


Fig. 3 (a)-(f) Time dependence of fluorescence reviving process of **1** from 0 to 15 min in darkness (without UV irradiation), the process took place after irradiation of visible light for *ca.* 5 min and kept in darkness as time elapsed; Images of switching process of **1** within lysosomes in A549 cell lines: (g) switch-off state: irradiation of Vis-light for *ca.* 5min erases the red fluorescence; (h) switch-on state: placed in darkness for *ca.* 15 min makes the red fluorescence revive.

Subsequently, exploration of **1** as a switchable sensor to track lysosomes was carried out by incubating A549 cells. Firstly, a time course ring-open process of **1** was conducted within cells. After treatment with visible light for *ca.* 5 min, no obvious fluorescence was detected by laser confocal microscopy, which suggested that **1** were present in the ring-closed form upon illumination. Then, the images were recorded as time elapsed. Interestingly, the A549 cells were highlighted with red fluorescence and became increasingly brighter as the time elapsed. After the cells being kept in darkness for *ca.* 15 min, the red fluorescence of **1** almost revived (Fig. 3a-f). After that, the photoswitchable lysosomal tracking was performed with successive processes of irradiation of Vis-light for *ca.* 5 min and kept in darkness for *ca.* 15 min. As shown in Fig. 3g-3h and S11(ESI†), the reversible off-on fluorescence imaging of targeted cells could be repeated at least eight times within cells with little photobleaching, which was attributed to the acidic atmosphere induced ring-opening instead of an UV light induced process. In this case, the harm to live cell caused by UV-light can be readily avoided. Furthermore, the time-lapse ring-open process made **1** a potential candidate for real-time dynamic monitoring of lysosome.

For potential applications, the cell toxicity of **1** towards A549 and HeLa cell lines was measured using a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Fig. S12(ESI†), incubated with a significantly higher concentration of 40 μM for 12 h, cell viabilities were still nearly 85%, indicating that **1** showed low cytotoxicity towards live cells. This bodes well for the utility of this peptide probe, particularly in live cell imaging applications for lysosomal tracking. To further explore the cellular entry pathway of **1**, staining with **1** was investigated under conditions of different temperatures. At 4 °C, active cellular uptake of **1** by A549 cells was blocked (Fig. S9a-c, ESI†). In contrast, at 37 °C, uptake of **1** into the cells and subsequent staining of lysosomes was clearly observed (Fig. S9d-f, ESI†), suggesting that cellular uptake of **1** occurred in an energy-dependent fashion, most likely via endocytosis. Hence, peptide **1** was efficiently taken up by cells, which was largely attributed to the specific lysine-rich sequence and the excellent water solubility.

In summary, we have demonstrated that peptide **1** with spiropyran units can be used as a switchable sensor to reversibly track lysosomes in live cell lines in real-time. Upon accumulation in intracellular lysosomes, the low pH of lysosomal lumen promotes the ring-opening of the spiropyran units, which, as a result, makes it possible for **1** to serve as a lysosomal sensor. Moreover, as the process can be tuned by pH and visible light, it enables us to reversibly label lysosomes, which has been realized here for the first time to the best of our knowledge. Furthermore, as peptide **1** does not possess any general cytotoxicity, it might be of interest for applications in tumor diagnosis and therapy.

Notes and references

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