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

Lyosomes, 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). Lyosomes 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. However, lyosomal malfunctions can lead to different pathophysiological conditions, especially to cancer-related diseases. Thus, effective strategies to visualize lyosomes of cancer cells are of significant interest to prevent or treat tumor invasion and metastasis and will pave the way for lyosome-related disease diagnosis and therapy.

Very recently, large efforts have been devoted to the development of fluorescent sensors employed to study lyososomal functions in live cells, which includes polymers, nanoparticles, modified quantum dots, and macromolecules, such as dextran labeled with a fluorophore. Although these sensors can be exploited to track the intracellular lyosomes efficiently, they also exhibit the following limitations: (i) it is difficult to target them to specific lyosomal 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 lyosomes with good solubility, high efficiency and excellent biocompatibility. In addition, sensors capable of reversibly monitoring lyosomal imaging are also desirable. This ideal probe, however, has rarely been reported in the field of lyosomes.

Up to now, spiropyran derivatives were widely applied from data storage to optical switching, chemical sensing, molecular machines and logic gate, based on the reversible structural isomerization from a colorless spiro-form (spiropyran, SP) to a colored open-form (merocyanine, Me) by UV/Vis light illumination. However, pH-active spiropyran based sensor used to track intracellular lyosomes 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 lyosomal tracking. Therefore, we expected to employ a spiropyran fluorophore with a structurally simpler lysine-rich cationic peptide to be used for lyosomal tracking. This photoswitch would allow us to track and recognize lyosomes with high signal-to-noise upon spectroscopic changes imposed by pH-changes in aqueous solution.

In this work, a bis-spiropyran functionalized peptide 1 (Scheme 1a) was specifically designed in anticipation of the tracking of lyosome upon the protonation of spiropyran moieties as well as the electrostatic interaction of positive charged lysine residues with negative charged lyosomal membrane. This peptide has been proved to serve as a pH and light dual-responsive photoswitch, in which the pH-induced ring open process was thoroughly studied. Moreover, it was well employed to reversible lyosomal tracking within live cells. Peptide 1 was synthesized using a microwave-assisted solid-phase peptide method that commonly used for peptide-based biosensors, which was isolated by preparative HPLC on a reversed-phase C-18 column in 99 % purity and 25 % yield, respectively (ESI†).
bathochromic shift from 410 nm to 514 nm concomitantly (Fig. 1).

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 (McH¹), which is consistent with a previous research. 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. 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 peptide 1 from pH 2 to 10 (Fig. S6), (details are not given). In contrast, the 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†).

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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). Amplified imaging of A549 cells shown in Fig. 2a 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 5 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 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 within cells was conducted. 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 high 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-h 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