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ARTICLE TYPE

A turn-on fluorescent probe for imaging lysosomal hydrogen sulfide in living cells

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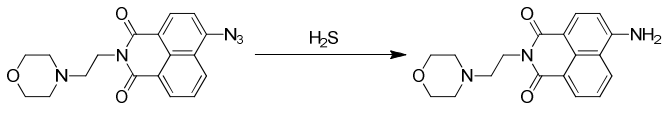
Hydrogen sulfide (H_2S) is an endothelia gasotransmitter which has been extensively studied recently in various physiological processes. H_2S can induce lysosomal membrane destabilization leading to an autophagic event of precipitation apoptosis coupled with calpain activation, thus ensuring cellular demise. In this study, we developed a lysosome-targetable fluorescent probe for the recognition of H_2S with considerable fluorescence enhancement. Through introducing a lysosome-targetable group 4-(2-aminoethyl)-morpholine into the H_2S probe *N*-imide termus of 4-azide-1,8-naphthalimide, the new compound **Lyso-AFP** can recognize H_2S in lysosomes. This probe emerges as a more biocompatible analysis tool with low poison by-product than reported H_2S fluorescent probes.

Introduction

Fluorescence imaging in living cells is a powerful technique to study biological systems in vivo.[1, 2] By attaching a sub-cellular organelle specific group, fluorescent probes are able to detect target analytes and reveal a diverse range of physical/chemical properties in specific regions of a cell.[3-5] Lysosomes are spherical-shaped, catabolic organelles with an acidic interior (pH 4.0-6.0). They are vital for degradation and recycling of macromolecules delivered by phagocytosis, endocytosis, and autophagy. Lysosomes were considered merely to be cellular waste bags for a long time. Nowadays, lysosomes are recognized as advanced organelles involved in many cellular processes and are considered crucial regulators of cell homeostasis.[6, 7] Evidences have shown that lysosomes are related to the pathogenesis of diseases such as storage disorders, cancer, neurodegenerative disorders, and cardiovascular diseases.[6] So real-time detection and imaging of lysosomal analytes would aid the understanding of intracellular reaction kinetics and mechanisms, and further assist the development of diagnostic and treatment strategies. In recent years, some fluorescent probes have been reported to stain lysosomes[8-13] or image lysosomal pH,[14-16] Ca^{2+},[17] Zn^{2+},[18-20] Cu^{2+},[21, 22] NO,[23] H_2O_2,[24] legumain,[25] viscosity,[26] and phospholipase A2 activity.[27]

Hydrogen sulfide (H_2S), a well known pungent gas, is generated endogenously in mammalian tissues from the amino acids cysteine and homocysteine by three enzymes including cystathionine-β-lyase (CSE) , cystathionine-γ-synthetase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST)[28]. Nowadays, H_2S has been considered as a crucial signal molecule in nervous system, cardiovascular system, and inflammatory system. In the nervous system, H_2S has been found to modulate neuronal transmission by facilitating the induction of hippocampal long term potential (LTP)[29]. In the cardiovascular system, H_2S can relax muscle and regulate blood pressure[29]. H_2S is also believed to be related with some diseases like Alzheimer's disease[30], Down's syndrome[31], diabetes[32] and liver cirrhosis[33]. Furthermore, H_2S also functions in lysosome organelles. H_2S can induce cell death in association with the activation of calpain proteases and lysosomal destabilization along with the release of lysosomal proteases[34]. Therefore, high sensitive and selective techniques for detecting H_2S in lysosomes seem to be great valuable.

In our previous work, we reported the first lysosome-targetable fluorescent probe **Lyso-NHS** for imaging H_2S in living cells based on the thiolysis of dinitrophenyl ether.[35] In consideration of potential toxicity of the leaving dinitrophenyl thiol ether to biological system, a much more biocompatible fluorescent probe for lysosomal H_2S imaging is desired. Chang et al.[36] and Wang[37] et al. pioneered an approach of using the reduction of azide with H_2S to amine to sense H_2S, which releases a much non-cytotoxic N_2 as the byproduct. This approach has been expanded to design various fluorescent probes for H_2S by altering fluorophores.[38-43] In this work, we introduced a lysosome-targetable group 4-(2-aminoethyl)-morpholine[23] into the N-imide termus of 4-azide-1,8-naphthalimide to yield the fluorescent probe **Lyso-AFP** (Scheme 1), and studied its properties in lysosomal H_2S imaging.



Scheme 1. Mechanism of H_2S sensing by **Lyso-AFP**.

Experimental section

Materials and methods

Unless otherwise noted, materials were obtained from Aldrich

and were used without further purification. The synthesis of compound *N*-(Morpholinoethylamino)-4-bromo-1,8-Naphthalimide (**3**) was according to the published procedure.[35] Melting points were measured using a Büchi 530 melting point apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker 400 MHz. Chemical shifts were given in ppm and coupling constants in Hz. UV-Vis absorption spectra were obtained on Agilent Cary 60 UV-Vis Spectrophotometer. Fluorescence emission spectra were obtained using Cary Eclipse Fluorescence Spectrophotometer.

Synthesis and characterization of Lyso-AFP

A solution of sodium azide 350 mg (5.4 mmol) in 5 mL water was added dropwise into the solution of compound **3** (2.0 g, 5.1 mmol) in 30 mL DMF. The reaction mixture was stirred at 100˚C for 8 hours. Then the mixture was added into ice water. Yellow solid was collected and dried in a vacuum drying oven, which was purified by silica gel column chromatography (CH_2Cl_2:MeOH = 100:1) to afford compound **Lyso-AFP** (1.7 g) in 90% yield. Mp: 144-146 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (d, *J* = 12.0 Hz, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.43 (d, *J* = 8.0 Hz, 1H), 7.74 (d, *J* = 8.0 Hz, 1H), 7.47 (t, *J* = 8.0 Hz, 1H), 4.33 (t, *J* =6.0 Hz, 2H), 3.68 (t, *J* = 4.0 Hz, 4H), 2.70 (t, *J* = 6.0 Hz, 2H), 2.59 (*J* =4.0 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.0, 163.6, 143.5, 132.2, 131.7, 129.2, 128.8, 126.9, 124.4, 122.6, 118.9, 114.7, 67.1, 56.2, 53.8, 37.3. HRMS (ESI) calcd for $C_{18}H_{18}N_5O_3$ [MH+] 352.1404, found 352.1419.

Synthesis and characterization of compound 1.

Lyso-AFP (100 mg, 0.28 mmol) was added to a round bottom flask under argon and dissolved in 50 mL acetonitrile. Then NaHS (24 mg, 0.43 mmol) was added slowly and the mixture was allowed to stir at room temperature for 24 h. The solvent was removed under reduced pressure and the resulted brown solid was purified by silica gel column chromatography (CH_2Cl_2:MeOH = 50:1) to afford compound 1 in 83% yield. ^{1}H NMR (400 MHz, DMSO) δ 8.61 (d, *J* = 8.4 Hz, 1H), 8.43 (d, *J* = 8.4 Hz, 1H), 8.19 (d, *J* = 8.4 Hz, 1H), 7.76 – 7.59 (m, 1H), 7.42 (s, 2H), 6.85 (d, *J* = 8.4 Hz, 1H), 4.15 (t, *J* = 7.0 Hz, 2H), 3.53 (t, *J* = 8.0 Hz, 4H), 2.53 (t, *J* = 7.0 Hz, 2H), 2.46 (t, *J* = 8.4 Hz, 4H). HRMS (ESI) calcd for $C_{18}H_{20}N_3O_3$ [MH+] 326.1499, found 326.1524.

Culture of Hela cells and fluorescent imaging

Hela was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were seeded in 24-well flat-bottomed plates and then incubated for 48 h at 37 ℃ under 5% CO_2. **Lyso-AFP** (5 μM) was then added to the cells and incubation for 30 min followed. Neutral Red (NR) (2 μM) was next added to co-stain the cells for 10 min. Then, the cells were washed three times with phosphate-buffered saline (PBS). Fluorescence imaging was observed under a confocal microscopy (Olympus FV1000) with a 60×objective lens.

Results and discussion

Effect of pH on the fluorescence of Lyso-AFP

In lysosomes, in order to maitain the pH in range of 4.0-6.0, Vacuolar H+-ATPases are usually responsible for transport of protons[44]. So, to monitor H_2S in lysosomes, the probe should remain stable in acidic environment with no fluorescence response. Firstly, we investigated behavior of **Lyso-AFP** in a wide range of pH values in acetonitrile-water (50:50) solution (Fig 1). What we can see from the fluorescence spectrum of **Lyso-AFP** is that the probe exhibited a weak emission band with a maximum at 535 nm. Hence, the stable fluorescence of **Lyso-AFP** in the pH range 3.0-12.0 can provide its application in monitoring intracellular H_2S without being affected by changes in physiological pH values.

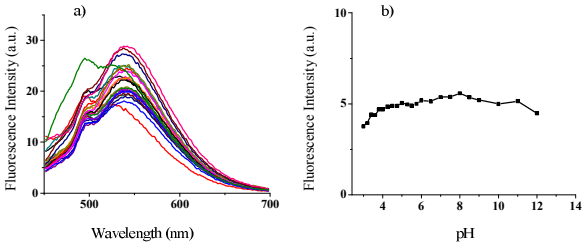


Fig 1. Influence of pH on the fluorescence of **Lyso-AFP** in aqueous solution. Excitation wavelength is 426 nm. **[Lyso-AFP]** = 10 μM.

Characterization of fluorescent probe Lyso-AFP for H_2S

Firstly, the absorption spectrum of **Lyso-AFP** in aqueous solution (CH_3CN:HEPES = 5:5, pH = 7.4) shows an absorption band at 370 nm. When we add NaHS (10 μM) to the solution of the probe, the band centered at 370 nm displays sharp decrease in absorbance along with the appearance of a new absorption band at longer wavelength (426 nm) (Fig 2) which is visible to the naked eye with a clear colour change from colourless to pale yellow. And, it indicates if the concentration of NaHS reach 20 equiv, the reaction can be completely finished in minutes.

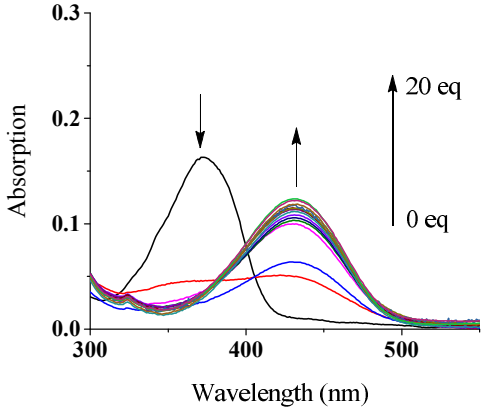


Fig 2. UV-Vis absorption spectra of 10 μM compound **Lyso-AFP** in the presence of 0-20 equiv of H_2S in aqueous solution (CH_3CN:HEPES = 50:50, pH = 7.4).

We then tested the fluorescence properties of **Lyso-AFP** for sensing H_2S in aqueous solution (CH_3CN:HEPES = 50:50, pH = 7.4) (Fig 3). Spectra were recorded after the addition of H_2S from 0 to 40 min, and the results showed that the reaction was completed within 30 min (Fig 3a, 3b). Notably, the background fluorescence of **Lyso-AFP** is very weak (Φ = 0.012), and within

minutes a high fluorescence (Φ = 0.263) increase is observed which signals the reaction of **Lyso-AFP** with H_2S (Fig 3b); therefore, the timescale may allow **Lyso-AFP** to sense H_2S in real-time intracellular imaging. Furthermore, when H_2S was added progressively from 0 equiv to 30 equiv to the solution of **Lyso-AFP**, the fluorescence intensity at 535 nm was dramatically increased due to the reduction of azide group to amine by H_2S (Scheme 1). From fig 3d, it was also found that if the concentration of NaHS was over 20 equiv, the reaction can be completed. Therefore, we used 20 equiv of H_2S to examine the performance of **Lyso-AFP** in all following experiments.

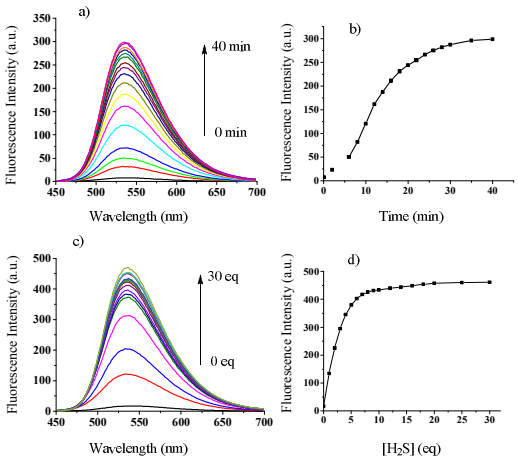


Figure 3. a) Time dependence of fluorescence profiles of **Lyso-AFP** (10 μM) with 20 equiv H_2S. (NaHS was dissolved in water in the concentration of 1 mM). Excitation at 426 nm. b) Time dependence of fluorescence intensity of **Lyso-AFP** (10 μM) at 535 nm with 20 equiv H_2S. c) Fluorescent emission spectra of 10 μM compound **Lyso-AFP** in the presence of 0-30 equiv of H_2S in in aqueous solution (CH_3CN:HEPES = 50:50, pH = 7.4). d) Fluorescence intensity of 10 μM compound **Lyso-AFP** in the presence of 0-30 equiv of H_2S.

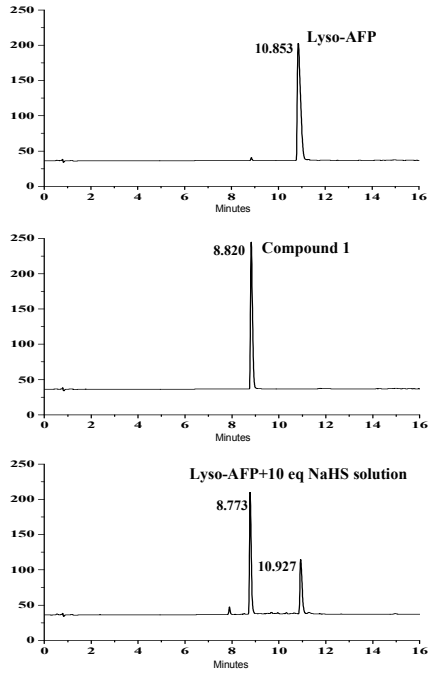


Fig 4. HPLC chromatogram in the reaction of **Lyso-AFP** (1 mg/mL) with NaHS (10 equiv) in CH_3CN.

In addition, compound **1** was synthesized independently and was confirmed by ^{1}H-NMR and HRMS (Fig S4). And, the HPLC retention time of compound **1** is the same as the reduction product of **Lyso-AFP** (Fig 4), which indicates that **1** is responsible for the fluorescence enhancement at 535 nm.

Generally, realizing higher selectivity toward a specific analyte over other potential competing species is necessary for a fluorescence chemosensor. So, we explored the fluorescence spectral changes of **Lyso-AFP** (10 μM) incubated with various cations, anions and sulfur-containing analytes in aqueous solutions (CH_3CN:HEPES = 50:50, pH = 7.4, Fig 5). By comparision, when **Lyso-AFP** was treated with 20 equiv NaHS, a great fluorescent enhancement was observed. While, the addition of 20 equiv of Na^+, K^+, Mg^{2+}, Ca^{2+}, Ag^+, Zn^{2+}, F^-, Cl^-, Br^-, ClO_4^-, HCO_3^-, NO_3^-, NO_2^-, PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, $P_2O_7^{4-}$, $S_2O_3^{2-}$, $S_2O_4^{2-}$, $S_2O_5^{2-}$, $S_2O_8^{2-}$, SO_3^-, N_3^-, SCN^-, CO_3^{2-}, CH_3COO^-, SO_4^{2-}, HSO_4^-, Citrate, Hydrogen citrate, Dihydrogen citrate, Ascorbic acid, L-Cysteine, Homocysteine, L-Glutathione and *N*-Acetyl-L-Cysteine exerted a negligible change on the fluorescence response for **Lyso-AFP**. In this regard, **Lyso-AFP** can be considered as a good OFF-ON chemosensor for specific recognition of H_2S.

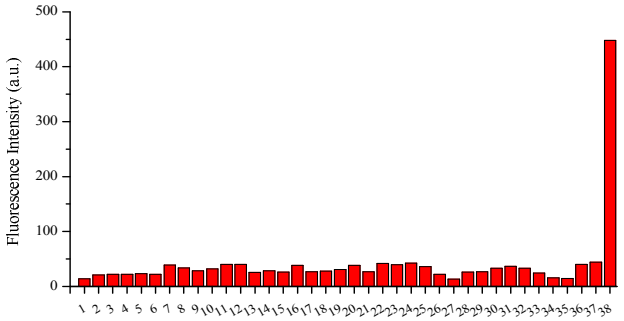


Fig 5. Fluorescence responses of 10 μM **Lyso-AFP** to various analytes in aqueous solution (CH_3CN:HEPES = 5:5, pH = 7.4, 37 ℃). Excitation at 426 nm. Bars represent the final fluorescence intensity of **Lyso-AFP** with 1 mM analytes over the original emission of free **Lyso-AFP**. 1) free **Lyso-AFP**; 2) Ag^+; 3) K^+; 4) Na^+; 5) Mg^{2+}; 6) Ca^{2+}; 7) Zn^{2+}; 8) F^-; 9) Cl^-; 10) Br^-; 11) CH_3COO^-; 12) ClO_4^-; 13) CO_3^{2-}; 14) HCO_3^-; (15) NO_3^-; (16) NO_2^-; (17) PO_4^{3-}; 18) HPO_4^{2-}; 19) $H_2PO_4^-$; 20) $P_2O_7^{4-}$; 21) SO_4^{2-}; 22) HSO_4^-; 23) SO_3^{2-}; 24) $S_2O_3^{2-}$; 25) $S_2O_4^{2-}$; 26) $S_2O_5^{2-}$; 27) $S_2O_8^{2-}$; 28) SCN^-; 29) N_3^-; 30) Citrate; 31) Hydrogen citrate; 32) Dihydrogen citrate; 33) Ascorbic acid; 34) L-Cysteine; 35) Homocysteine; 36) L-Glutathione; 37) N-Acetyl-L-Cysteine; 38) HS^-.

Imaging of lysosomal H_2S with Lyso-APF

We next sought to apply **Lyso-AFP** to the detection of H_2S in Hela cells. When incubated with 5 μM **Lyso-AFP** for 30 min, the cells were washed with phosphate buffered saline (PBS) (pH 7.4) to remove excess of **Lyso-AFP**. Then, Hela cells exhibited no fluorescence seen from the confocal image (Fig 6a). While after incubated with 50 μM NaHS for 4 min, the cells displayed enhanced green fluorescence (Fig 6b). Another 4 min later, a

higher turn-on fluorescence response was observed (Fig 6c). Moreover, the fluorescence intensity reached the maximum in 20 min. All these experiments demonstrated the potential biological application of **Lyso-AFP** for imaging H_2S in living cells.

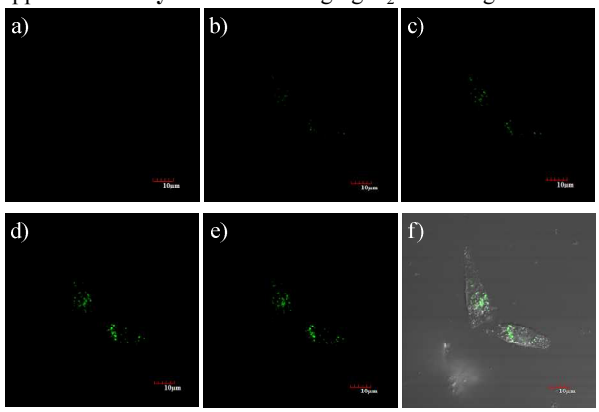


Fig 6. Time-dependent exogenous H_2S released from NaHS (20 μM) in Hela cells stained with **Lyso-AFP** (5.0 μM) at 37 ℃ a) 0 min; b) 4 min; c) 8 min; d) 16 min; e) 20 min; f) merged images of e) and bright field. Scale bars = 10 μm.

In order to confirm whether **Lyso-AFP** can specifically stain the lysosomes, Neutral Red (2 μM), a commercially available probe for lysosome, was used to stain the Hela cells at the same time. The yellow parts in fig 7c represent the colocalization **1** and **NR**. The fluorescence patterns of **1** and **NR** signals merged very well, which indicated the fluorescence response of **Lyso-AFP** to H_2S was mainly located in the lysosomes. The intensity profiles of the linear regions of interest across Hela cells stained with **Lyso-AFP** and **NR** also displayed in close synchrony (Fig 7e). The high Pearson's coefficient and overlap coefficient are 0.970 and 0.971, respectively (Fig 7f). The cytotoxicity of **Lyso-AFP** was examined toward Hela cells by a MTT assay (Fig S1). The results showed that > 90% Hela cells survived after 24 h (5.0 μM **Lyso-AFP** incubation), demonstrating that **Lyso-AFP** was of low toxicity toward cultured cell lines.

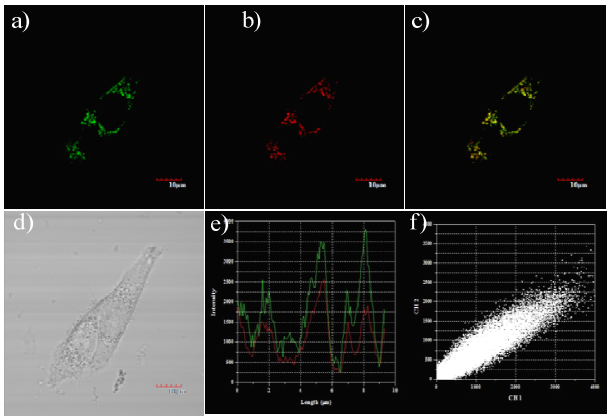


Figure 7. **Lyso-AFP** co-localizes to lysosomes in Hela cells. a) 5.0 μM **Lyso-AFP** with 50 μM of H_2S incubated 20 min at 37 ℃ (Channel 1: λ_{ex} = 458 nm, λ_{em} = 510 - 559 nm). b) 2.0 μM **NR** (Channel 2: λ_{ex} = 559 nm, λ_{em} = 561-610 nm). c) Merged images of a) and b). d) Bright field image. e) Intensity profile of regions of interest (ROI) across Helo cells. f) Intensity correlation plot of dyes **Lyso-AFP** and **NR**. Scale bars = 10 μm.

Conclusion

In summary, we reported a novel fluorescence probe based on 1,8-naphthalimide derivatives which can be used for imaging H_2S in lysosomes. The rapid reduction of azide to amine makes **Lyso-AFP** convert to compound **1** with strong green fluorescence in minutes. Compared with previous work, the probe has better biocompatibility due to its low toxicity, safer byproduct, and insensitivity to pH over lysosomal pH range. Besides, **Lyso-AFP** was proved to be highly selective for H_2S and it did not response to other biological mercaptan. Its potential application in living cells encourages us to actively pursue much more biocompatible fluorescent probes for imaging H_2S in different organelles.

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Notes and references

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‡Footnotes should appear here.

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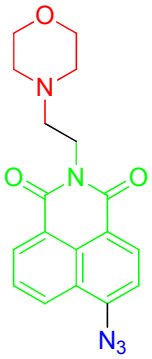
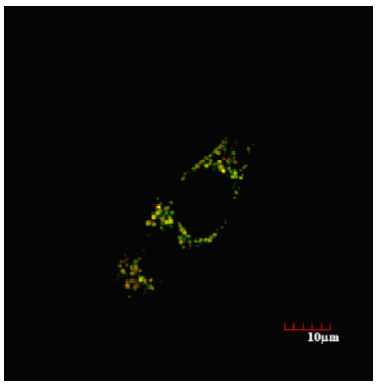


Lysosome-targetable Group

Fluorescence Signal Group

H2S Sensing Group



Lyso-AFP

A 1,8-naphthalimide-derived fluorescent probe for lysosomal H_2S based on the reduction of azide is reported.