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A NIR-Emitting Lysosome-Targeting Probe with Large Stokes Shift via Coupling Cyanine and Excited-State Intramolecular Proton Transfer

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Abstract

An NIR-emitting probe ($\lambda_{em} \sim 700$ nm) with a large Stokes shift ($\lambda \sim 234$ nm) is synthesized by using excited-state intramolecular proton transfer (ESIPT). The phenolic proton, which controls ESIPT, acts as a switch to give strong fluorescence at pH ≈ 5 . The probe can selectively show lysosome organelles, therefore leading to a lysosome probe without exhibiting “an alkalinizing effect”.

Graphical abstract

A NIR-emitting probe with a large Stokes shift ($\lambda \sim 234$ nm) can selectively show lysosome organelles without exhibiting “an alkalinizing effect”.

Lysosomes are membrane-bound organelles, which contain degradative enzymes to digest a variety of proteins, carbohydrates, and nucleic acids that are brought into the cell.¹ To metabolize these complex molecules, a variety of digestive enzymes function optimally under rather acidic conditions (pH ≈ 5). When one of the normally present digestive enzymes is lacking (or not working), it is known as lysosomal storage diseases.^{1,2} Because of the importance of lysosomes to cell function and human health, there are strong interests in developing fluorescent probes for visualization of subcellular organelles.³ Most lysosome probes include organic amines to facilitate their selectivity, which induce an increase in lysosomal pH (an alkalinizing effect),⁴ causing adverse effect on cell activity. It remains a challenge to develop a fluorophore which can target lysosome without perturbing the pH.

[†]Electronic Supplementary Information (ESI) available: Synthesis and characterization of **1**, **3** and **8**; ¹H NMR and Mass spectral data; additional UV-vis absorption and fluorescence data. See DOI: 10.1039/x0xx00000x

Lysosome probes with fluorescence in the near-infrared (NIR) region are also desirable, as NIR signals will not be interfered by autofluorescence. Current molecular design in NIR probes uses BODIPY,^{5,6,7} benzofuran,⁸ rhodamine,⁹ and cyanine¹⁰ as fluorophores, which typically exhibit small Stokes' shift. In addition, most NIR lysosome probes are including an amine group in the probe design.^{5,6,8,9}

Fluorescent dyes with excited-state intramolecular proton transfer (ESIPT)¹¹ have emerged to be an attractive mechanism for new probes¹² and optoelectronic materials.¹³ ESIPT-based fluorophores exhibit emission with large Stokes' shift (e.g. >150 nm). Among the reported ESIPT systems, 2-(2'-hydroxyphenyl)benzoxazole (HBO) and 2-(2'-hydroxyphenyl)benzothiazole (HBT) derivatives are frequently used sensor frames for cations,^{14,15,16} anions^{17,18} and binuclear assembly.¹⁹ However, the ESIPT emission of HBO or HBT derivatives is typically in blue,¹⁵ green,^{16,19} and orange-red color,^{18,20} with few giving NIR emission.²¹ It is highly desirable to develop ESIPT chromophores that give NIR-emission.

Cyanine dyes are one class of the common NIR-emitting fluorophores for biological imaging applications.²² For example, a cyanine dye with a benzothiazolium group is quite effective in shifting the absorption and emission to a longer wavelength.²³ However, the cyanine dyes exhibit a small Stokes' shift ($\lambda \approx 10\text{-}60$ nm).²⁴ Herein, we illustrate that a cyanine dye with large Stokes shift ($\lambda \approx 264$ nm) can be assembled by coupling two molecular segments (i.e. HBT and benzothiazolium cyanine), through sharing a *meta*-phenylene bridge (Scheme 1). Due to π -conjugation interruption at the *meta*-phenylene, the effective chromophores in **1** can be approximated by HBT **2** and cyanine **3** in the ground state.²⁵ As a consequence of ESIPT on HBT **2**, cyanine **1** gave NIR emission with a large Stokes' shift ($\lambda \approx 264$ nm). Remarkably, the ESIPT ON-OFF event occurs at an acidic pH, which could be used to sense lysosome organelles.

The desirable **1** was synthesized by reaction of aldehyde **4** with 3-ethyl-2-methylbenzo[d]thiazolium iodide (**5**) in 86% yield (Scheme 1). Benzothiazolium derivative **3** was also prepared to aid the spectroscopic study. ¹H NMR of **1** detected the resonance signal at ~ 13.4 ppm (Figure S3), indicating that the phenolic proton was involved in the intramolecular hydrogen bonding²⁶ that was necessary for proton transfer.

Cyanine **1** revealed the absorption peak at $\lambda_{\text{abs}} \sim 447$ nm and the emission peak at $\lambda_{\text{em}} \sim 681$ nm in CH₂Cl₂ (Figure 1), showing a very large Stokes' shift ($\lambda \approx 234$ nm). Cyanines **1** was assumed to have the same effective chromophore as **3**, on the basis of their almost identical λ_{abs} values. The observed large Stokes shift from **1**, therefore, must be due to the incorporation of HBT unit (**2**), which introduces the spectral feature associated with ESIPT mechanism. Lack of spectral overlap between the absorption and emission, as shown between 540–580 nm from **1**, is a typical feature observed from an ESIPT probe. Interestingly, compound **1** exhibited a much higher fluorescence quantum yield ($\phi_{\text{f}} \approx 0.1\text{-}0.3$) than **3** (Table 1). The results clearly indicated that inclusion of HBT segment had a significant impact on the fluorescence of cyanine segment by improving its Stokes' shift and quantum yield.

It is known that HBT **2** exhibits absorption $\lambda_{\text{abs}} \approx 343$ nm and a predominate emissions at $\lambda_{\text{em}} \approx 527$ nm (from its *keto* tautomer).²⁷ Lack of emission at about 527 nm from **1** (Figure 1a) indicated that any photons absorbed by HBT fragment **2** would be effectively transferred to the benzothiazolium segment **3**, as two chromophores are connected via sharing a *meta*-phenylene bridge for effective energy transfer (Scheme 1).²⁵ The observed emission from **1** ($\lambda_{\text{em}} = 681$ nm), however, could not be explained by a simple energy transfer, as it would lead to emission from **3** at ~ 583 nm.

On the basis of an unusually large Stokes' shift (~ 234 nm) from **1**, it was reasonable to speculate that the *enol* form **1a** was transformed into the corresponding *keto* tautomer **1b** via the ESIPT process upon photon absorption (Scheme 2). As a consequence of forming **1b**, the excited state generated an amine, which could serve as a terminal group for a polymethine dye, thereby enabling the NIR emission. The effective chromophore in **1b** ($\lambda_{\text{em}} \approx 681$ -705 nm) was quite comparable with the cyanine dye **6b** ($\lambda_{\text{abs}} \approx 639$ nm, $\lambda_{\text{em}} \approx 695$ nm).^{23,28} Since the *keto* tautomer **1b** was only formed in the excited state, it gave emission with a large Stokes' shift.

The assumption that **1a** has a shorter conjugation length than **1b** was examined by calculating their respective absorption spectra with Time-dependent self-consistent field (TD-SCF) method. The calculated absorption for the *enol* form **1a** ($\lambda_{\text{abs}} = 449$ nm, Figure S13) closely matched the experimental value ($\lambda_{\text{abs}} = 447$ nm) in CH_2CH_2 solvent. The calculated absorption for the *keto* form **1b** ($\lambda_{\text{abs}} = 580$ nm) was red-shifted drastically by ~ 130 nm to a longer wavelength. The results thus supported the assumption that ESIPT event was responsible for the large Stokes' shift from **1**, as the *keto* tautomer **1b** was only generated in the excited state.

It should be noted that the hydroxyl group in **1** is adjacent to the benzothiazol-2-yl group for ESIPT. In order to verify the impact of ESIPT in **1**, we synthesized the isomer **8**, in which the hydroxyl group was moved away from the benzothiazol-2-yl group and no longer capable of forming intramolecular hydrogen bonding. ^1H NMR showed that the phenolic proton of **8** was at $\delta \sim 11.7$ ppm (ESI Figure S7), significantly upfield shift from **1** ($\delta \sim 13.3$ ppm). While the absorption of **8** ($\lambda_{\text{abs}} = 456$ nm) (Fig. 2) was red-shifted from **1** ($\lambda_{\text{abs}} = 447$ nm), the emission of **8** was significantly blue-shifted from **1** (by ~ 70 nm, $\lambda_{\text{em}} = 613$ nm). Lack of spectral separation between the absorption and fluorescence of **8** clearly pointed out that the ESIPT was responsible for the large Stokes' shift in **1**.

As shown in Scheme 2, formation of **1b** enables a strong intramolecular charge transfer (ICT). In order to evaluate the extent of ICT effect, the solution of **1** in a quartz tube (with 3 mm inside diameter) was quickly cooled by immersing the sample solution into liquid nitrogen in a quartz Dewar. The spectra were then acquired as the temperature was gradually raised (within a few hours). At the extremely low temperature (at -188°C), the molecule gave the emission peak at 635 nm. When the temperature was warmed to -121°C , the emission peak exhibited a notable red-shift, which completed in a narrow temperature range (from -121°C to -101°C) in EtOH (m.p -112°C). When further raising temperature to room temperature (from -101°C up), there was no obvious spectral shift, as both ESIPT and ICT were permitted. It should be noted that ESIPT was not expected to exhibit the spectral shift

upon cooling in liquid N₂.²⁹ Therefore, the observed large spectral shift ($\lambda_{em} \approx 77$ nm) was attributed to ICT within the formed *keto* tautomer **1b**, as the bond reorganization and rotation became possible at a higher temperature (necessary to accommodate the changes in the excited state).

Optical absorption and fluorescence of **1** was examined in different buffer solutions to examine its pH Response (Figure 4, and ESI Fig. S14(b)). Compound **1** was nearly non-fluorescent in basic solution, attributing to deprotonation of phenolic proton (as absorption λ_{max} was red-shifted from ~ 447 nm to ~ 565 nm). Interestingly, the fluorescence of **1** became highly fluorescent in acidic aqueous solution (pH < 6). The observation further illustrated that the emission of **1** was controlled by ESIPT, which was consistent with the large difference observed from **1** and **3** (Figure 1) as the latter does not exhibit ESIPT. The pK_a of **1** was determined to be 5.72 (ESI Figure S14). At high pH (e.g. pH ≈ 11), there is an increase in fluorescence after 780 nm, which could be attributed to the formation of anionic species.

Cell Imaging

Cytotoxicity of **1** was examined by using an MTT assay, showing that the half maximal inhibitory concentration (IC₅₀) for **1** was 31 μ M in normal human lung fibroblast cells. Low toxicity of **1** and its large fluorescence response to pH ($\approx 4-6$) encouraged us to examine the staining in normal human lung fibroblast (CC-2512) cells. When the cells were incubated with **1** for 1 hour and imaged by confocal microscopy, fluorescence signals were observed primarily from the perinuclear region (Fig. 5A), indicating that the dye might be selectively binding to intracellular organelles. In order to confirm the intracellular location and distribution of **1**, we examined the colocalization of our dye with the commercial LysoTracker Red DND-99. Staining with both **1** and the commercial LysoTracker revealed the same pattern (Fig. 5A and 5B), indicating that **1** was selective to lysosome organelles.

Strong fluorescence signals from **1** could be attributed to the significant acidity (pH ~ 4.5) in lysosomes, in comparison with extracellular region (pH ~ 7.2) and other intracellular organelles (endoplasmic reticulum pH ~ 7.2 , Golgi-network pH $\sim 6-6.7$, endosome pH $\sim 5.5-6.5$).³⁰ It should be noted that the compound **1** was not expected to exhibit any “alkaline effect” as its pK_a (≈ 5.72) is significantly different from that of a trialkyl amine (pK_a ≈ 10.75).³¹ Although cyanine **1** does not have a weakly basic amino group, the observed contrast of lysosome imaging by using **1** was comparable to that by using the commercial LysoTracker (Fig. 5A and 5B). The result suggests that **1** might be accumulated in the lysosomes.

In summary, an NIR-emitting dye **1** ($\lambda_{em} \sim 700$ nm) with a large Stokes' shift ($\lambda \approx 234$ nm) has been synthesized by integrating the ESIPT mechanism into a benzothiazolium-derived cyanine unit. The study illustrates that ESIPT can be used as an effective mechanism to increase the Stokes' shift of cyanine dyes, while giving NIR emission. In addition to its large Stokes' shift and NIR emission, cyanine **1** also exhibits a large fluorescence in acidic pH. The ability of **1** to diffuse into lysosome organelles, in addition to its enhanced fluorescence in acidic environment, leads to a NIR-emitting lysosome probe that is of low toxicity in live

cells. It is assumed that **1** can freely diffuse through the organelle membranes. The fluorescence on/off switching of **1** is controlled by a reversible phenol/phenoxide interconversion, which occurs under a rather acidic condition (without exhibiting “an alkalizing effect”). This is different from the typical amine-containing lysotrackers, whose accumulation increases pH of lysosomes through a buffering effect. Therefore, probe **1** could offer significant advantages for *in vivo* imaging of live cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Absorbance (broken line) and fluorescence (solid line) of **1** (a) and **3** (b) in CH_2Cl_2 (10 μM). Fluorescence spectra were acquired by excitation at 449 nm. The inset in (b) shows the fluorescent image for solution **1** and **3** under UV excitation (365 nm).

Figure 2. Absorbance (broken line) and fluorescence (solid line) of **8** in CH_2Cl_2 ($10\ \mu\text{M}$). Excitation at 456 nm.

Figure 3. Fluorescence spectra of **1** in EtOH (3 μ M) at different temperatures. Inset shows the response of emission peak λ_{em} to temperature.

Figure 4. Fluorescence of **1** in different pH in water (excitation at 415nm). Inset on the right shows the plot of fluorescence intensity at different pH.

Figure 5.

Images of a normal human lung fibroblast (NHLF) stained with (A) LysoTracker (1 μM), (B) compound **1** (0.5 μM), (C) overlap under magnification of 100x and (D) Enclosed portion of C which is digitally enhanced by 367x. Excitation for **1** is 405 nm with 680–720 nm filter for emission, and the LysoTracker has an excitation/emission of 577/590 nm.

Scheme 1.
Synthesis of cyanine derivatives **1** and **3**.

Scheme 2.

Structures of *enol* and *keto* tautomers, and cyanine **6**. The dotted squares indicate the effective chromophore in **1**.

Table 1

Photophysical properties of **1** and **3** in different solvents.

solvent	1			3		
	λ_{abs}	λ_{em}	ϕ_{f}	λ_{abs}	λ_{em}	ϕ_{f}
CH ₂ Cl ₂	447	681	0.32	497	583	0.009
THF	435	705	0.062	434	581	0.006
MeCN	423	705	0.21	416	566	0.008
MeOH	423	705	0.15	425	581	0.007
water	384	700	0.05	408	587	0.003