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pH-Responsive Cy5 dyes having nucleophilic substituents for molecular imaging

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ABSTRACT

pH-Responsive fluorescent pentamethine cyanine (Cy5) derivatives having nucleophilic substituents were synthesized. Cy5 derivatives **1-O** having mercaptopropyl, hydroxypropyl, and aminopropyl groups on an indole nitrogen atom showed pH-dependent equilibria between a fluorescent open-ring structure (**1-O**) and a non-fluorescent closed-ring structure (**1-C**) in pH ranges 3–7, 6–7.5, and 8–9, respectively. pH-Responsive dyes **1a-C** having a 1,3-thiazinane structure were easily internalized into A549 cells and converted to open-ring structure **1a-O** in response to the relatively low pH of acidic compartments in the cells.

Introduction

Fluorescent dyes are one of the most powerful probes for real-time monitoring of an unknown bioactivity in living cells and tissues using optical imaging devices [1,2]. pH-Responsive fluorescent dyes are considered important for high-contrast visualization of diseased sites and monitoring proton concentration gradients, because abnormal pH values of intra- and extracellular microenvironments are associated with common diseases, including inflammatory disease, cancer, and Alzheimer's disease [3]. To date, many pH-responsive fluorescent dyes taking advantage of xanthene [4], boron-dipyrromethene [5], coumarin [6], and naphthalimide [7] core structures have been developed. Raymo and co-workers reported photoresponsive and/or pH-responsive cyanine dyes bearing a nucleophilic 4-nitrophenol-2-yl moiety as the sensor material [8]. For tumor-specific visualization, we developed pH-responsive near-infrared fluorescent cyanine dyes by introducing nucleophilic substituents into heptamethine cyanine (Cy7.5) dyes (Fig. 1a) [9]. Non-fluorescent dyes having a closed-ring structure are formed by the annulation of intramolecular nucleophilic moieties under conditions of high pH. By contrast, heteroatoms in the cyclic structures are efficiently protonated to form open-ring structures under conditions of low pH, leading to the recovery of fluorescence emission, even in living cells. Although the fluorescence under acidic conditions is suitable for detecting the slightly acidic extracellular microenvironment of solid tumor tissues, the fluorescence at 800–820 nm cannot be used to visualize pH-differences of organelles in cells, using a fluorescence microscope. In this context, we prepared pH-responsive fluorescent dyes **1-O**/**1-C** by introducing nucleophilic substituents into pentamethine cyanine (Cy5) dyes, which are one of the most reliable fluorescent dyes for cytological staining (Fig. 1b). Cy5 derivatives having aminopropyl and hydroxypropyl groups on an indole nitrogen atom were previously reported as fluorescent labelling agents of biomaterials, such as DNA and RNA, but their pH-responsiveness has not been noted (Fig. 1c) [10]. Because the pH of individual cellular organelles and compartments in cells varies [11], we have carefully investigated the pH-responsiveness of Cy5 derivatives **1-O**/**1-C** *in vitro*.

Results and discussion

A mixture of Cy5 derivatives **1-O** and **1-C** (**1-O**/**1-C**) having nucleophilic mercaptopropyl, hydroxypropyl, and aminopropyl groups (1,3-thiazinane, 1,3-oxazinane, and hexahydropyrimidine) were synthesized from 2,3,3-trimethyl-3*H*-indole (Scheme 1) (see Supporting Information). According to the literatures [12], *N*-substituted-3*H*-indoles **2a**–**c** were prepared by nucleophilic substitution of 2,3,3-trimethyl-3*H*-indole with corresponding alkyl halides. The condensation reaction of **2a**–**c** with the reactive cyanine derivative **3** [13] afforded protected Cy5 derivatives **4a**–**c** in 52–71% yields. After deprotection under basic conditions, **1-O**/**1-C** were obtained in 57–93% yields, **1-C** being the major component.

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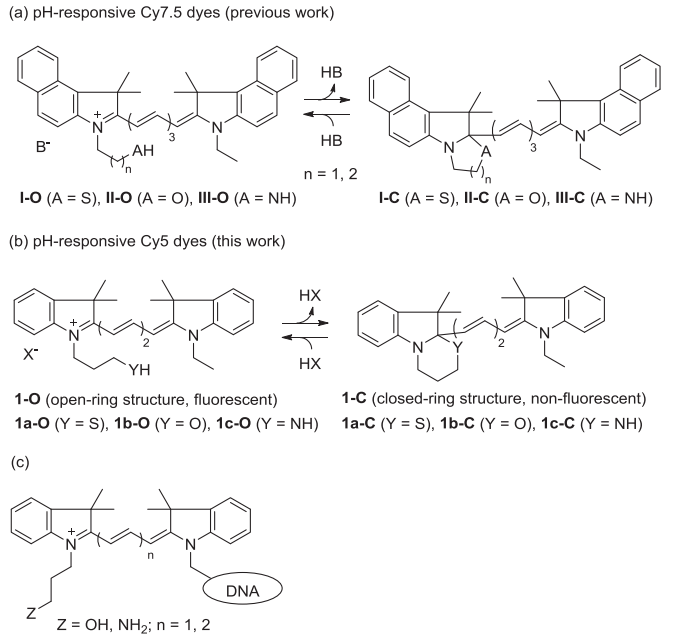
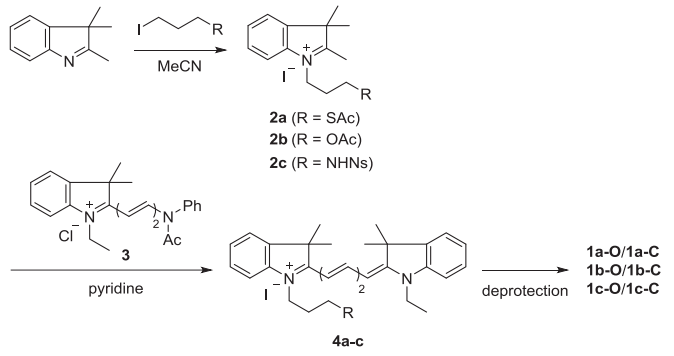


Fig. 1. (a) pH-Responsive Cy7.5 dyes (our previous work) and (b) pH-responsive Cy5 dyes (this work). Equilibrium between **1-O** having an open-ring structure and **1-C** having a closed-ring structure. (c) Cy3 and Cy5 derivatives as DNA labeling agents.



Scheme 1. Synthesis of pH-responsive Cy5 dyes **1-O/1-C**.

All dyes were assigned using high-resolution ESI mass spectra and NMR.

To evaluate the pH responsiveness of Cy5 derivatives **1-O**/**1-C**, UV-vis absorption and fluorescence spectra of **1-O**/**1-C** (5.0×10^{-6} M) in buffered aqueous solutions were measured under various pH conditions (Fig. 2). Because pH-responsive dyes **1-O**/**1-C** are insoluble in water, a buffered solution of dyes **1-O**/**1-C** with a surfactant was prepared and used for measurement. A buffered solution (3.0 mL) containing Triton X-100 (2.0×10^{-3} M) as a surfactant was incubated for 30 min at ambient temperature; then to this solution was added a solution of **1-O**/**1-C** in dimethyl sulfoxide (10 μL, 1.5×10^{-3} M). In the UV-vis absorption spectrum of **1a-O**/**1a-C** under neutral conditions, two signals were observed (Fig. 2a); one signal observed at 300–400 nm is assigned to be closed-ring structure **1a-C** having shorter π-conjugation, while the other signal at 600–700 nm is the open-ring structure **1a-O**, being similar to that of Cy5 dye **A** (Fig. 4a) [12a]. A time-dependent density functional theory calculation of closed-ring structure supports the assignment of UV-vis absorption signals (Fig. S9 in Supporting Information). As the pH of solutions decreased from 7 to 3, the absorption (300–400 nm) of **1a-C** decreased and the corresponding absorption (600–700 nm) of **1a-O** increased (Fig. 2a). The p*K*a of **1a-O**/**1a-C** is estimated at 5.2 (Fig. 3a), indicating that **1a-O**/**1a-C** is most suitable for detection of a slightly acidic environment, such as that found in late endosomes. In the case of **1b-O**/**1b-C**, the ring-opening reaction proceeded to form **1b-O** even under neutral conditions (pH 6–7.5) (Figs. 2b and 3a). Although the fluorescent **1c-O** having an open-ring structure was detected under high pH conditions, the ring-opening reaction of **1c-C** smoothly proceeded to form **1c-O** under slightly basic conditions (pH 8–9) (Figs. 2c and 3a). The pH-dependent change of fluorescence intensity of **1a-O**/**1a-C**, **1b-O**/**1b-C**, and **1c-O**/**1c-C** was observed under weakly acidic conditions (pH 4–7), neutral conditions (pH 6.5–8), and weakly basic conditions (pH 8–9), respectively (Figs. 2d–f and 3b). For both **1b-O**/**1b-C** and **1c-O**/**1c-C**, the pH-dependent change of absorbance at 650 nm also took place in the range of pH 10–12, along with a similar change of fluorescence intensity. This indicates that a part of **1-C** converted to **1-O** even under basic conditions, although the mechanism is unclear. The difference in pH-responsive ranges of **1a-O**/**1a-C**, **1b-O**/**1b-C**, and **1c-O**/**1c-C** is caused by differences in the basicity of 1,3-thiazinane, 1,3-oxazinane, and hexahydropyrimidine. The p*K*a of **1a-O**/**1a-C**, **1b-O**/**1b-C**, and **1c-O**/**1c-C** in absorption (fluorescence) is 5.2 (5.4), 6.5 (7.3), and 8.3 (8.1), respectively (Table S1). The p*K*a of Cy7.5 derivatives, **I-O**/**I-C**, **II-O**/**II-C**, and **III-O**/**III-C** (Fig 1a), is 4.3 (4.9), 6.1 (6.9), and 8.1 (9.9), respectively [9]. These values are similar to Cy5 derivatives **1-O**/**1-C**. The reversibility between open-ring structure **1a-O** and closed-ring structure **1a-C** was evaluated, while the pH values of a buffered solution of **1a-O**/**1a-C** (pH 6.9) between pH 3 and pH 7 were changed several times (Fig. S5 in Supporting Information). The rapid and repeatable on/off switching of absorbance at 650 nm was observed without substantial change of intensity in absorbance. The photostabilities of Cy5 derivatives **1a-O** and **4a** were evaluated (Figs. S6 and S7). It was found that Cy5 dye **4a** is more stable than commercially available Cy7.5 dye, indocyanine green, under photoirradiation. In the case of **1a-O**, the photo-induced ring-opening of **1a-C** was observed within 10 min [8a].

To determine the pH responsiveness of dyes in living cells, we incubated A549 cells with **1a-O**/**1a-C** or fluorescent Cy5 dye **A** at 4 °C (on ice, to prevent endocytosis) or 37 °C for 4 h in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS, 10%). We monitored the time-dependent change of absorbance intensities during incubation at 37 °C and 4 °C (Figs. 4b and c). The Cy5 dye **A** exhibited constant absorption intensities during the incubation at 37 °C, but a gradual increase of absorption intensity of **1a-O** was observed during the incubation at 37 °C. This indicates that **1a-C** responded to the acidic environment in A549 cells, thereby increasing the amount of open-ring structure **1a-O** in that environment. By contrast, we did not observe a significant change of absorption maxima during the incubation of A549 cells with dyes **A** and **1a-C** at 4 °C. This finding suggests that the increase of absorption intensities of **1a-O** at 37 °C is caused by the cellular activity, such as endocytosis. Because dye **1a-C** is not transferred to acidic endosomes at 4 °C, no structural change of **1a-C** occurred. After the incubation at 37 °C for 4 h, cells were washed with phosphate buffered saline (PBS) and the absorbance of the open-ring structure in cells was measured. As shown in Fig. 4d, dyes **A** and **1a-O** internalized into cells were clearly detected. Taking these results into consideration, dye **1a-C**, which is internalized into cells, responded to the acidic compartments in cells and smoothly transformed to the open-ring structure **1a-O** during the incubation at 37 °C. We also monitored the time-dependent change of fluorescence intensities during incubation at 37 °C and 4 °C (Figs. 4e and f). In the case of **1a-O**/**1a-C**, the fluorescence of **1a-O** increased after incubation at 37 °C for 1 h. Although the absorbance of **1a-O** continued to increase during