

# A New Water-Soluble Near-Neutral Ratiometric Fluorescent pH Indicator

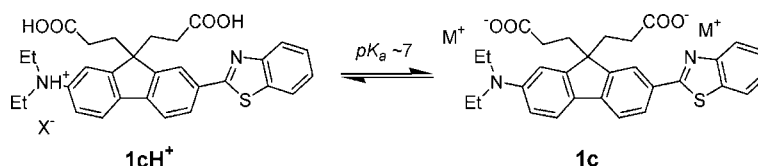
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## ABSTRACT



Donor- $\pi$ -acceptor fluorene derivative **1c** is a near-neutral pH indicator whose  $pK_a$  of  $\sim 7.0$  was determined by both absorption and fluorescence methods. **1c** satisfies important criteria for a sensitive ratiometric fluorescent pH indicator with a distinctive isoemissive point, good dispersion in cell cytosol, and low cytotoxicity. Furthermore, its 2PA cross section of 100 GM in its neutral form suggests its potential in two-photon fluorescence imaging applications.

The dynamics of intracellular pH is believed to be crucial for understanding the regulation mechanism of many physiological functions in cells.<sup>1</sup> Of the several methods available to determine pH, optical methods have several advantages including rapid response time, high signal-to-noise ratio, noninvasiveness, and excellent pH sensitivity. Since the first use of a trapped intracellular pH probe, 6-carboxyfluorescein, was described by Thomas et al.,<sup>2,3</sup> a number of pH indicators have been reported with a variety of properties.<sup>1,4–11</sup> In order

to quantitatively measure pH, the  $pK_a$  of the indicator needs to match the pH of the experimental system. Since the pH in the cell cytosol is between 6.8 and 7.4, there is tremendous interest in developing efficient near-neutral fluorescent pH indicators. Fluorescent indicators with ratiometric properties are highly desirable since the ratio of the fluorescence intensity at peak wavelength vs insensitive isoemissive wavelength is constant regardless of the change of fluorophore concentration by photobleaching or change of the external environment such as ion concentration. Here, we report a sensitive near-neutral ratiometric pH indicator based on a new class of fluorene derivatives.

Fluorene derivatives are known for exhibiting high fluorescence quantum yields and excellent photostability.<sup>12,13</sup> Recently, fluorene-based conjugated compounds have also been reported as excellent two-photon fluorescent (2PF) materials, in which they possess high two-photon absorption

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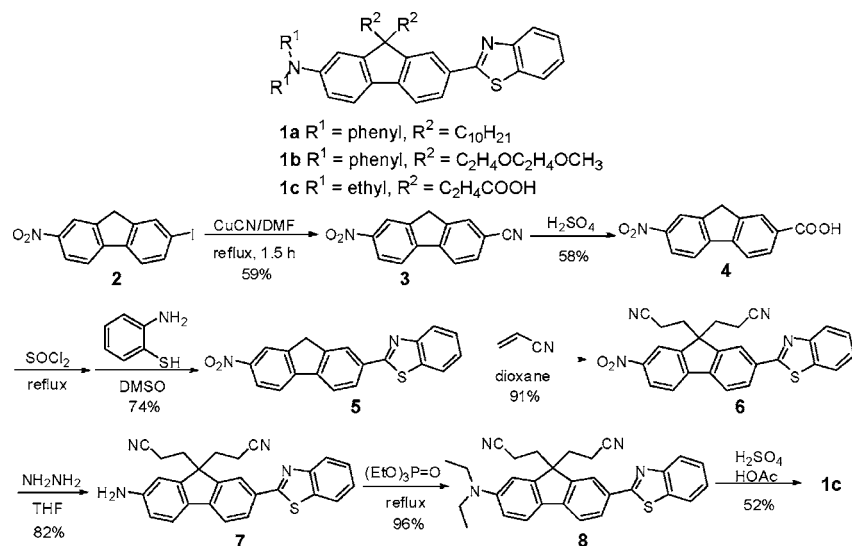
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**Scheme 1.** Structures of Fluorene Derivatives **1a–c** and Synthesis of Fluorescent Probe **1c**



(2PA) cross sections.<sup>14–18</sup> As part of our efforts to develop high-efficiency two-photon absorbing fluorene derivatives as novel fluorescent biomarkers, we found D- $\pi$ -A type compounds 7-(benzo[d]thiazol-2-yl)-*N,N*-diphenyl-9*H*-fluoren-2-amine, such as chromophores **1a** and **1b** (Scheme 1), have utility for one-photon and two-photon fluorescence cell imaging, with near unity fluorescence quantum yields and reasonable 2PA cross sections, i.e.,  $\sim 100 \text{ GM}$  ( $1 \text{ GM} = 10^{-50} \text{ cm}^4 \text{ s photon}^{-1}$ ).<sup>19,20</sup> However, these two compounds are rather hydrophobic, thereby limiting their applications in aqueous biological environments. Thus, compound **1c** was specifically designed to improve the water solubility by introduction of the dipropionic carboxylic acid at the 9-position of the fluorene ring, and by replacement of the diphenylamino group in **1a** and **1b** with diethylamino group, resulting in a  $\text{pK}_a$  close to 7.0. Therefore, the potential of **1c** as a near-neutral ratiometric pH fluorescent indicator was investigated and is reported herein. The 2PF properties of **1c** were also investigated due to its potential in 2PF biological imaging.

The synthetic scheme for probe **1c** is shown in Scheme 1. Converting the iodo group in **2** into cyano afforded precursor **3** that was easily hydrolyzed to carboxylic acid **4**. The carboxylic acid was then transformed to the acid chloride in situ, and reacted with aminothiophenol in DMSO to form benzothiazole derivative **5**. Two propionitrile groups were

then introduced in **5** by a Michael reaction. Subsequent reduction of nitro to amine, diethylation of the primary amine with triethylphosphate, and hydrolysis of the nitrile groups afforded the final product **1c** in good overall yield. All compounds were fully characterized by NMR and elemental analysis (or HRMS in the case of compounds with carboxylic acid moieties).

As expected, compound **1c** exhibits reasonable water solubility, i.e.,  $>10^{-4} \text{ M}$  at pH 1–7 and  $>10^{-3} \text{ M}$  at pH 7–12. At pH 4, the absorption and emission maxima of protonated **1cH**<sup>+</sup> are 341 and 391 nm, respectively (Figure 1A), with a fluorescence quantum yield of 0.21. The 2PA cross section of the **1cH**<sup>+</sup> was low in the excitation wavelength range of 570–750 nm, which may be attributed to very strong electron-withdrawing nature of the protonated nitrogen of the diethylamino group (see the  $\text{pK}_a$  measurement). In this A- $\pi$ -A type structure, the absorption wavelength is expected to shift to shorter wavelength and the single-photon excitation allowed  $\text{S}_0$ – $\text{S}_1$  transition is not allowed by two-photon excitation.<sup>21</sup> In contrast, at pH 10 buffer, where the neutral form of **1c** exists, the absorption maximum underwent a bathochromic shift of 41 to 382 nm, and the fluorescence quantum yield increased to 0.56 (Figure 1B). Significantly, a dramatic increase of the 2PA cross section to ca. 100 GM at the excitation wavelength of 770 nm was observed. The formally 2PA forbidden single-photon allowed  $\text{S}_0$ – $\text{S}_1$  transition of **1c** (382 nm) is accessible by 2PA due to the relaxed D- $\pi$ -A symmetry of the molecule.<sup>21</sup> Even though 2PA was measured at pH 10, as can be seen in the inset of Figure 2, the deprotonated (high 2PA) form predominates at pH 7.5 and above, making this probe especially attractive for 2PF imaging.

The pH-dependent absorption spectra are presented in Figure 2 for titration in aqueous buffer. With the increase in

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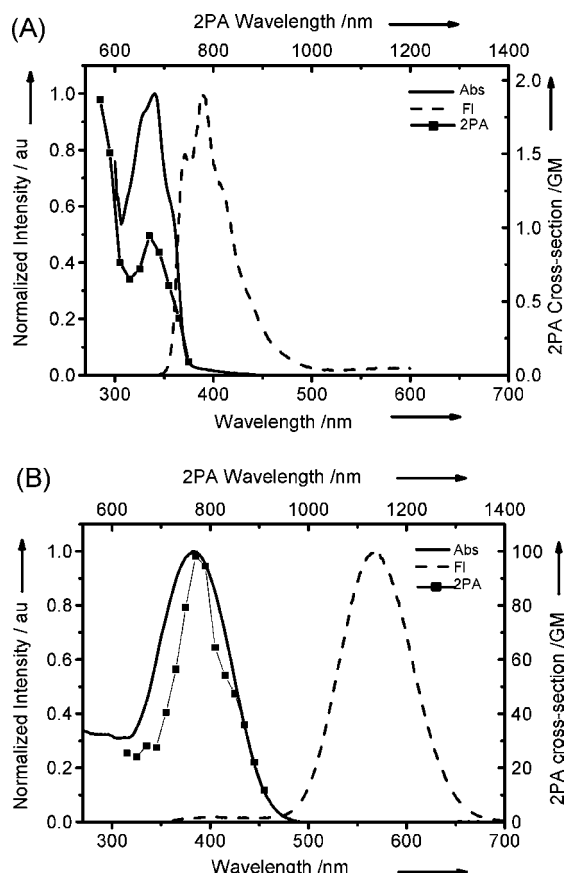
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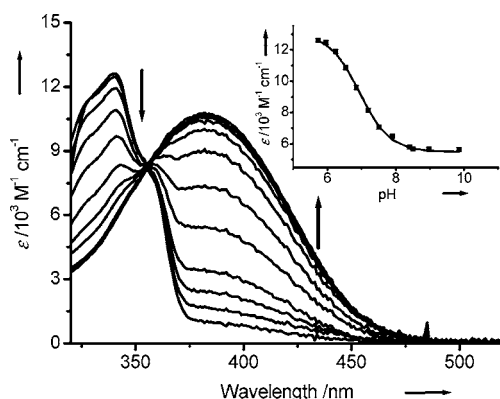
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**Figure 1.** Absorption (solid line), emission (dashed line), and two-photon absorption (squares) spectra of (A) protonated form **1cH<sup>+</sup>** (in pH 4 buffer) and (B) neutral form **1c** (in pH 10 buffer). The absorption and fluorescence spectra are normalized.

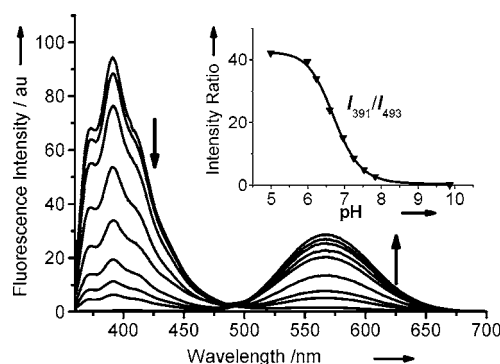
pH, the absorption peak at 341 nm, attributed to the protonated form of **1c**, becomes weak, and the absorption



**Figure 2.** pH dependence of the absorption spectra of dye **1c** in buffer at concentration of 0.01 mM with arrows indicating the change of the absorption intensities with pH increase. (Inset) Nonlinear fitting of the pH-dependent extinction coefficients at 341 nm.

peak of the neutral form at 382 nm evolved with a well-defined isosbestic point at 355 nm. It is known that the basicity of the diethylaminobenzene nitrogen<sup>22</sup> is significantly higher than that of the benzothiazole nitrogen.<sup>23,24</sup> Hence, the structure of the protonated form of **1c** is assigned as protonation at the diethylamino site. The inset in Figure 2 shows the results of the nonlinear regression of the  $\lambda$  at 341 nm according to a literature method,<sup>25</sup> affording a  $\text{pK}_a$  value of  $6.95 \pm 0.01$ . The  $\text{pK}_a$  value may also be calculated from the pH dependence of the total integrated fluorescence emission of **1c** excited at 340 and 382 nm, taking the advantage of the well-separated emission bands of the two forms. Similar nonlinear regression analysis yielded nearly same  $\text{pK}_a$  value ( $6.96 \pm 0.04$ ).

When **1c** was excited at the wavelength of the absorption isosbestic point (355 nm), fluorescence emission from both protonated and neutral forms was observed, as shown in Figure 3. The characteristic isoemission point at 493 nm makes **1c** an excellent ratiometric pH indicator.



**Figure 3.** pH-dependence of the fluorescence intensity of **1c** in buffer ( $10^{-5} \text{ M}$ ) excited at 355 nm (absorption isobestic point wavelength in Figure 2) with arrows indicating the change of the fluorescence intensities with pH increase. (Inset) Ratiometric calibration curve of  $I_{391}/I_{493}$  (intensity at 391 nm vs intensity at isoemissive point 493 nm).

For intracellular applications, one major concern for the probes is the cell permeability of the indicator. Therefore, **1c** was incubated with NT2 (NTERA-2 cl.D1 [NT2/D1]) cells, and subjected to two-photon fluorescence imaging (Figure 4). From this cursory study, it was clear the dye had good permeability and dispersed well in the cytosol. In addition, this promising result implies that **1c** may also serve as a precursor of 2PF amine reactive probes that can be readily activated by esterification of the carboxylic acid group using succinimide hydroxide.

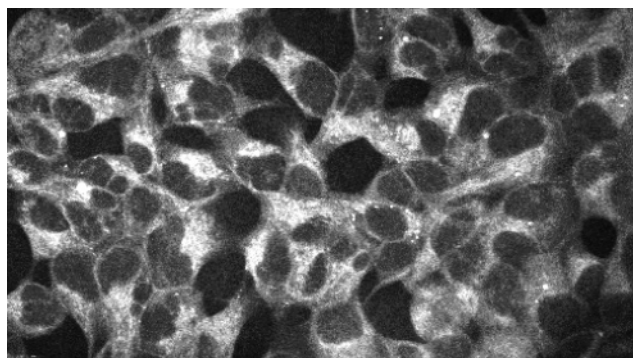
The cytotoxic effect of **1c** on proliferating cells is another parameter of primary interest, particularly for live-cell

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**Figure 4.** 2PF image of NT2 cells incubated with **1c** excited at 795 nm (164 fs, 76 MHz).

fluorescence imaging applications. An Alamar Blue (AB) reduction analysis<sup>26</sup> was used to assess cytotoxic effects of **1c** on proliferating NT2 cells. The cells were treated with different concentrations of compound **1c** (0.1–100  $\mu$ M) dissolved in buffer, and were also treated with 10% AB solution. The observed fluorescence intensity of AB reduction by cells treated with various doses of **1c** was similar to that observed for cells untreated with any fluorene compound

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(control) after 48 h, indicating low toxicity of **1c** over a relatively wide concentration range.

In summary, probe **1c** was synthesized as a near-neutral fluorescent pH indicator with  $pK_a$  of 6.96, confirmed by both absorption and fluorescence methods. The distinctive iso-emissive point observed in the fluorescence spectra at different pHs, good dispersion in cell cytosol, and low cytotoxicity indicates that **1c** satisfies important criteria for an excellent one-photon ratiometric fluorescent pH indicator. Furthermore, its high 2PA cross section in the deprotonated form at pH > 7 suggests its potential in other 2PF imaging techniques (a subject of future investigation).

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**Supporting Information Available:** Detailed synthesis procedures and characterization of the compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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