Tuning the Hoechst Dye into Color-changing Fluorescent pH Indicator in an Acidic Range

Shinsuke Sando,* Atsushi Narita, Masayoshi Hayami, and Yasuhiro Aoyama*
Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510

(Received November 27, 2007; CL-071315; E-mail: ssando@sbchem.kyoto-u.ac.jp; aoyamay@sbchem.kyoto-u.ac.jp)

We developed a new fluorescent pH indicator from a common Hoechst 33258 fluorescent dye. Unlike the parent compound, the improved Hoechst probe, wherein two methyl substituents were introduced into the terminal phenol ring, is fluorescent in an aqueous solution at neutral pH, and showed a unidirectional wavelength shift in the fluorescence emission spectra on lowering the pH. Thus, the probe can be used as a blue/green color-changing fluorescent pH indicator in an acidic range.

Among the reported non-invasive pH sensing methods, fluorescence-based pH monitoring techniques have been extensively employed in recent years, especially for analysis of intracellular pH change,[1,2] because of their high sensitivity and good spatial resolution. Typical fluorescent probes detect changes in micro-environmental pH conditions by virtue of their pH-dependent fluorescence intensities at a fixed wavelength. However, intensity-based evaluation can occasionally lead to ambiguous results because the probe concentration/distribution cannot be accurately defined. Therefore, there has been a recent demand for fluorescent pH probes that respond to pH changes by a wavelength shift in the emission spectra, and not by an intensity change.[3] This allows us to negate the inaccurate concentration/distribution factors by ratiometric measurements at different wavelengths. Here, we report a new scaffold for fluorescent pH indicator, which was developed from a well-known live cell fluorescent DNA imager, with a pH-dependent shift in emission wavelengths in an acidic range.

Hoechst dyes are well-known fluorophores composed of bisbenzimidazole chromophore.[4] A representative Hoechst 33258 (Figure 1, R = H) is live cell-permeable and preferably binds to AT-rich domain of double-stranded DNA (dsDNA). The fluorescence emission of Hoechst 33258 is suppressed in an aqueous media at neutral pH. However, it is enhanced in a low dielectric environment such as minor groove of the AT-rich dsDNAs.[5] Therefore, Hoechst dyes can be used as a fluorescent nucleus-stainer of live cells.[6] In addition, the fluorescence of the Hoechst is known to be sensitive to the changes in environmental pH.[7] Figure 2a shows the fluorescence spectra of Hoechst 33258 (2 μM, excitation at 345 nm at 25 °C) in 100 mM sodium acetate or citrate buffer in an acidic pH range (3.0–7.0). As shown in the Figure 2a, the fluorescence of the Hoechst 33258 changed depending on pH conditions, demonstrating its potential utility as a fluorescent pH indicator. However, the dye requires improvement for the following reasons. The pH change only causes an intensity change, while the emission wavelength practically remains unchanged ($\lambda_{max} = 495$ nm at pH 5.0). Second, the observed fluorescence change is not unidirectional. The fluorescence intensity was observed to increase from pH 7.0 to 5.0, and then decreased as the pH was further lowered. In addition, the intrinsic affinity of Hoechst 33258 to the AT-rich dsDNA target could lead to an erroneous signal when applied to biological samples containing genomic DNAs.

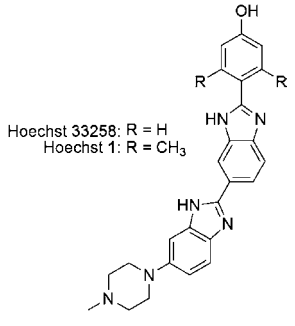

Figure 1. Chemical structures of Hoechst derivatives.

Given the above-mentioned limitations of the Hoechst 33258 dye, the objective was to identify the minimum modifications that can alter the conventional Hoechst dye into a new scaffold for fluorescent pH sensor. Molecular modeling suggests that just two *ortho*-methyl substituents on the terminal phenol ring lead to a conformational change, wherein the terminal phenol ring prefers almost perpendicular to the benzimidazole chromophore possibly with a restricted rotation around the benzimidazole–phenol linkage.[8] This is probably because of the steric repulsions of the methyl groups (Hoechst derivative **1**[9] in Figure 1), which is in marked contrast with conventional Hoechst 33258 that can form a more planar conformation. Since the induced-planar structure of the Hoechst 33258 dye is crucial to fit along the cleft of the minor groove of dsDNA,[10] the non-planar conformation of Hoechst **1** is thought to inhibit its binding to the original target. Also, even if the terminal phenol ring of Hoechst **1** could form a planar structure and could bind along the minor groove of DNA, the methyl substituent on the terminal phenol ring seems to be sterically repulsive against nucleobases in the groove of the resulting complex.[11] That was the case. Hoechst **1** lost its strong binding affinity to the original AT-rich dsDNA target, as revealed by the thermal denaturation study of the dsDNA in the presence of the Hoechst dyes.[12] In addition, a recent report suggested that such intramolecular twisting or restriction of rotation could affect the photochemical properties of the Hoechst dye.[13] Hoechst **1** showed an absorption maxima at 325 nm with a molar extinction coefficient of 3.45×10^4 M^{-1} cm^{-1} at pH 7, which is slightly blue-shifted relative to that (≈340 nm) of the original Hoechst 33258[7c] (molar extinction coefficients of Hoechst **1** at pH 3–7, see Table S1).[15] Interestingly, however, there is a dramatic difference in the fluorescence behavior. Hoechst **1** restored its fluorescence intensity (fluorescence quantum yield $\Phi_F = 0.241$[14]) in an aqueous media at neutral pH. When the pH of the aqueous solution was changed from a neutral to acidic pH, i.e., from 7.0 to 3.0, the fluorescence

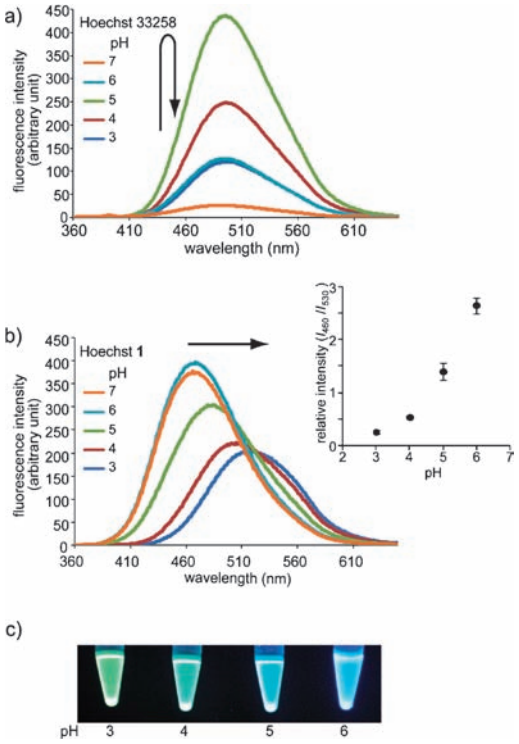

Figure 2. Fluorescence spectra of (a) Hoechst 33258 (2 μM, excitation at 345 nm) and (b) Hoechst derivative **1** (2 μM, excitation at 345 nm) in 100 mM sodium acetate or citrate buffer (pH 3, 4, 5, 6, and 7) at 25 °C. Inset shows relative fluorescence intensity (I_{460}/I_{530}, taken from the data in Figure 2b) of Hoechst derivative **1** as a function of pH. Error bars are standard deviations of three independent measurements. (c) Fluorescence image of Hoechst derivative **1** (12.5 mg/mL, excited with a 366 nm transilluminator) in the buffer (pH 3, 4, 5, and 6) containing 100 mM NaCl.

spectra of Hoechst **1** gradually shifted from $\lambda_{max} = 469$ nm to $\lambda_{max} = 521$ nm, a shift range of 52 nm (Figure 2b). Unlike Hoechst 33258, the pH-dependent changes in fluorescence were unidirectional, indicating that the Hoechst **1** probe could be used as a ratiometric fluorescent probe (i.e., at the ratio of 460/530 nm, shown in Figure 2b inset) for monitoring pH that is at least in the range 3–6. Because of the pH-dependent emission maxima, the pH of the solution could be visualized in color by a simple photo-irradiation of the Hoechst **1** dye in the solution. As shown in Figure 2c, the fluorescent color of the Hoechst **1** solution, irradiated with a 366 nm trans-illuminator, changed from blue (pH 6) to pale green (pH 3), thus, allowing us to use the dye as a color-based pH sensing device in an acidic range.

In conclusion, we report a new fluorescent pH probe with a unidirectional shift in the emission spectra upon change of pH in an acidic range. From a photodynamic point of view, mechanistic details for the present pH-dependence remain to be further clarified. However, it is interesting that the new pH probe was developed from a well-known fluorescent DNA-imager by a simple modification of the terminal phenol ring. The present results reveal a hidden potential of the conventional fluorescent dyes used in live-cells, and encourage further improvements and enhanced applications of these common but modified fluorophores.

We thank Mr. Kazumasa Ogawa for his contribution in an early stage of this work. This work was supported by an Industrial Technology Research Grant Program of the New Energy and Industrial Technology Development Organization (NEDO) of Japan and in part by JSPS. AN acknowledges the fellowship from JSPS.

References and Notes

1 R. P. Haugland, *The Handbook. A Guide to Fluorescent Probes and Labeling Technologies*, 10th ed., ed. by M. T. Z. Spence, Invitrogen, **2005**, p. 935.
2 T. Yeung, N. Touret, S. Grinstein, *Curr. Opin. Microbiol.* **2005**, *8*, 350.
3 Fluorescent pH indicator for ratiometric detection, for example: a) Y. Ando, S. Iino, K. Yamada, K. Umezawa, N. Iwasawa, D. Citterio, K. Suzuki, *Sens. Actuators, B* **2007**, *121*, 74. b) F. Gao, L. Tang, L. Dai, L. Wang, *Spectrochim. Acta, Part A* **2007**, *67*, 517. c) S. Charier, O. Ruel, J.-B. Baudin, D. Alcor, J.-F. Allemand, A. Meglio, L. Jullien, B. Valuer, *Chem.—Eur. J.* **2006**, *12*, 1097. d) C.-G. Niu, X.-Q. Gui, G.-M. Zeng, A.-L. Guan, P.-F. Gao, P.-Z. Qin, *Anal. Bioanal. Chem.* **2005**, *383*, 349. e) H. R. Kermis, Y. Kostov, P. Harms, G. Rao, *Biotechnol. Prog.* **2002**, *18*, 1047. f) K. P. McNamara, T. Nguyen, G. Dumitrascu, J. Ji, N. Rosenzweig, Z. Rosenzweig, *Anal. Chem.* **2001**, *73*, 3240. g) J. E. Whitaker, R. P. Haugland, F. G. Prendergast, *Anal. Biochem.* **1991**, *194*, 330 and references therein.
4 S. A. Latt, G. Stetten, *J. Histochem. Cytochem.* **1976**, *24*, 24 and references therein.
5 R. Jin, K. J. Breslauer, *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8939.
6 R. M. Martin, H. Leonhardt, M. C. Cardoso, *Cytometry* **2005**, *67A*, 45.
7 a) L. Goracci, R. Germani, G. Savelli, D. M. Bassani, *ChemBioChem* **2005**, *6*, 197. b) H. Görner, *Photochem. Photobiol.* **2001**, *73*, 339. c) K. K. Kalninsh, D. V. Pestov, Y. K. Roshchina, *J. Photochem. Photobiol., A* **1994**, *83*, 39.
8 Molecular modeling study was carried out using a simplified Hoechst model (Figure S1).[15]
9 S. Sando, A. Narita, Y. Aoyama, *ChemBioChem* **2007**, *8*, 1795.
10 a) S. Neidle, *Nat. Prod. Rep.* **2001**, *18*, 291. b) M. C. Vega, M. Coll, C. Alemán, *Eur. J. Biochem.* **1996**, *239*, 376.
11 R. F. Martin, S. Broadhurst, M. E. Reum, C. J. Squire, G. R. Clark, P. N. Lobachevsky, J. M. White, C. Clark, D. Sy, M. Spotheim-Maurizot, D. P. Kelly, *Cancer Res.* **2004**, *64*, 1067.
12 The binding affinities of Hoechst 33258 and Hoechst **1** to AT-rich dsDNA (5′-CGAATTCG-3′, 2.075 μM), known target of Hoechst 33258, were estimated by measuring the increase in the melting temperature (ΔT_m) in the presence of these Hoechst derivatives (4.15 μM) in a 50 mM Tris-HCl buffer (pH 7.2) containing 0.1 M NaCl. ΔT_ms by Hoechst 33258 and Hoechst **1** were 11.7 and 1.0 °C, respectively, showing the reduced affinity of Hoechst **1** to the original AT-rich dsDNA target. Details are provided in Ref. 9.
13 D. Banerjee, S. K. Pal, *Chem. Phys. Lett.* **2006**, *432*, 257.
14 Fluorescence quantum yield was determined in 100 mM Tris-HCl buffer pH 7.2 using 9,10-diphenylanthracene in EtOH ($\Phi_F = 0.95$) as a standard.
15 Supporting Information is available electronically on the CSJ-Journal Web site; http://www.csj.jp/journals/chem-lett/.

Published on the web (Advance View) February 2, 2008; doi:10.1246/cl.2008.246