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Synthesis and photophysical properties of new SNARF derivatives as dual emission pH sensors

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ABSTRACT

We report the synthesis and properties of two new seminaphthorhodafluor (SNARF) derivatives, SNARF-F and SNARF-Cl. Both these derivatives exhibit typical red shifts of absorption and fluorescence, and higher cell permeability as compared to traditional SNARF, while the pH-dependent dual-emission characteristics are well retained. In particular, the lower pK_a (7.38) of SNARF-F makes it more suitable than traditional SNARF derivatives for intracellular applications.

Spatial and temporal observation of intracellular pH changes is crucial for understanding regulation mechanisms of various cellular physiologic functions. For such observations, fluorescent spectroscopy is particularly advantageous as compared to other measurement methods, such as the use of microelectrodes, NMR, and absorbance spectroscopy.[1] Accordingly, various fluorescent pH indicators have been developed and applied to monitor intracellular pH changes.[2] Among such indicators, seminaphthorhodafluor (SNARF) is especially useful as a pH indicator because of its unique fluorescent characteristics.[3] First, SNARF can be excited by visible light, reducing cell damage due to irradiation and circumventing some disadvantageous effects of intracellular autofluorescence. Second, SNARF has dual-emission properties for ratiometric measurement, reducing irrelevant parameters such as optical path length, local probe concentration, photobleaching, and leakage from cells (Fig. 1). However, it has been pointed out that the relatively the high pK_a of SNARF (~7.5) has limited its intracellular applications.[2,4] To overcome this disadvantage, SNARF derivatives possessing fluorinated xanthene rings and having lower pK_a values because of fluorine substitution have been synthesized.[4] In order to improve cell permeability of SNARF, cell-permeable SNARF prodrug derivatives have also been synthesized, wherein the phenolic group is protected by acetate or acetoxymethyl ester groups that function as esterase substrates. A complication with these prodrugs arises if the protected SNARF remains inside the cell, because SNARF derivatives retain the fluorescence of the acidic form and may result in incorrect pH measurements.[5] As we described previously, one of the solutions to overcome this drawback is to design a SNARF derivative, that is, nonfluorescent before hydrolysis.[6] Another solution would be the development

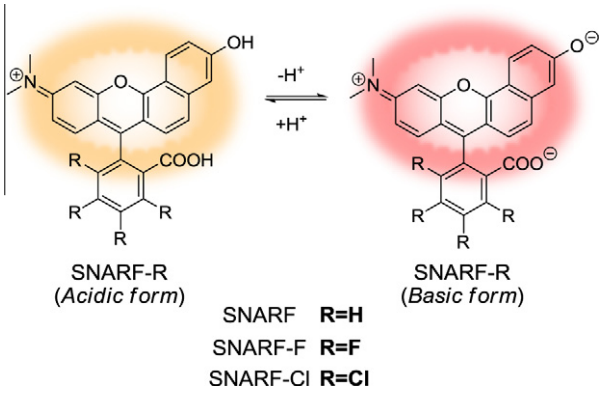

Figure 1. The equilibria and the structures of SNARF, SNARF-F and SNARF-Cl.

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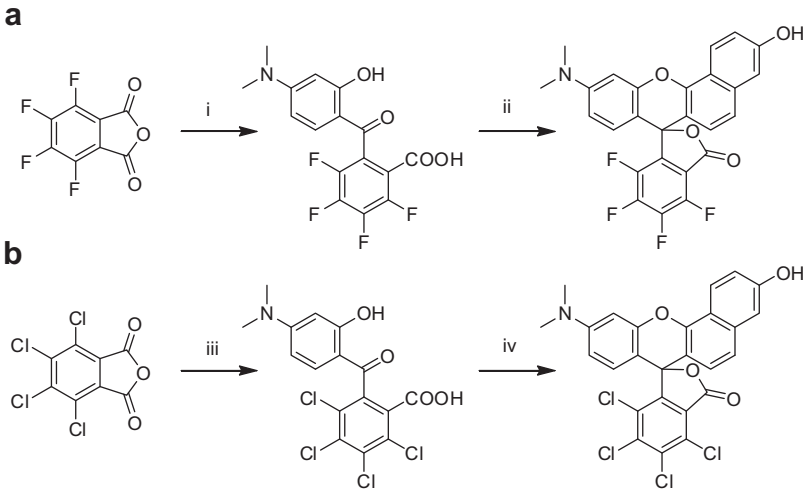

Scheme 1. Synthesis of (a) SNARF-F and (b) SNARF-Cl. Reaction conditions: (i) 3-dimethylaminophenol, toluene, reflux, yield: 55%; (ii) 1,6-dihydroxynaphtalene, $MeSO_3H$, reflux, yield: 33%; (iii) 3-dimethylaminophenol, toluene, reflux, yield: 36%; (iv) 1,6-dihydroxynaphtalene, $MeSO_3H$, reflux, yield: 48%.

Table 1
Photophysical properties of SNARF derivatives described in this study

SNARF derivatives	pH	$\lambda_{Abs,\ max}$ (ε M^{-1} cm^{-1})	$\lambda_{Abs,\ iso}$ (ε M^{-1} cm^{-1})	$\lambda_{em,\ max}$ (nm)	$\Phi(*10^{-2})$	c log P[9]	pK_a[a]
SNARF	5.0	515 nm (17,700) 544 nm (21,600)	534 nm (25,750)	583	3.0	4.99	7.62
	10.0	573 nm (44,100)		627	9.0		
SNARF-F	5.0	533 nm (25,600) 571 nm (28,900)	546 nm (23,100)	603	1.0	5.69	7.38
	10.0	603 nm (54,400)		650	2.0		
SNARF-Cl	5.0	539 nm (27,200) 574 nm (31,000)	532 nm (26,700)	603	1.0	7.29	7.90
	10.0	605 nm (47,500)		651	3.0		

[a] pK_a value were determined by UV spectrum change (see Supplementary data).

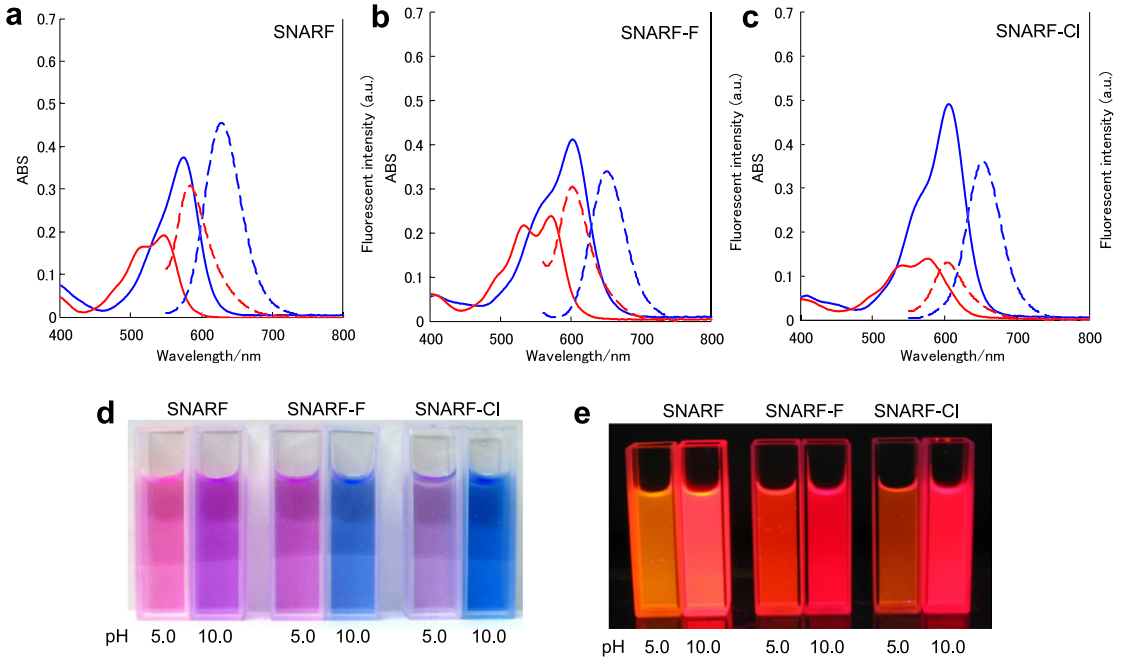

Figure 2. (a–c) Absorbance (plane line) and fluorescence (dash line) spectra of 10 μM of (a) SNARF, (b) SNARF-F and (c) SNARF-Cl at pH 5.0 (red) and pH10.0 (blue). (They were excited at the isosbestic point, respectively.) (d–e) Photos of (d) color and (e) fluorescence of SNARF (10 μM), SNARF-F (10 μM) and SNARF-Cl (10 μM) at pH 5.0 and pH 10.0.

of unprotected SNARF derivatives with higher inherent cell permeability.

These considerations motivated us to derivatize existing SNARF reagents by another strategy, that is, substitution of a hydrogen atom of the benzene moiety with a halogen atom, such as fluorine or chlorine, in order to increase hydrophobicity as well as to modulate photophysical properties. So far, there are only limited reports on the derivatization of benzo[*c*]xanthene fluorophores.[2,7] Herein, we report the synthesis and photophysical properties of the newly synthesized SNARF-F and SNARF-Cl indicators.

SNARF-F was synthesized from commercially available tetrafluorophthalic anhydride as shown in Scheme 1a. Reaction of 3-dimethylaminophenol with tetrafluorophthalic anhydride in toluene produced 2-carboxy-3,4,5,6-fluoro-3′-dimethylamino-2′-hydroxybenzophenone. Condensation of this product with 1,6-dihydroxynaphthalene afforded the desired SNARF-F dye. SNARF-Cl was synthesized from tetrachlorophthalic anhydride in a manner similar to the preparation of SNARF-F (Scheme 1b). Structures of all compounds were confirmed with ^{1}H NMR, high-MS, and elemental analysis (see Supplementary data).

The absorbance and fluorescence properties, pK_a, and calculated log *P* (c log *P*) of the SNARF derivatives are summarized in Table 1. In aqueous solution, SNARF produced maximal absorption and strong fluorescence at 544 and 583 nm, respectively, at pH 5.0, and 573 and 627 nm, respectively, at pH 10.0 (Fig. 2a). Notably, as compared to SNARF, the absorption and fluorescence of SNARF-F exhibited a typical red shift, that is, SNARF-F produced maximal absorption and strong fluorescence at 571 and 603 nm at pH 5.0, respectively, and maximal absorption strong fluorescence at 603 and 650 nm, respectively, at pH 10.0 (Fig. 2b). In the case of SNARF-Cl, a similar red shift was observed (Fig. 2c). The altered properties of absorption and fluorescence were clearly confirmed under bright-field and transilluminator observations (Fig. 2d and e). As described above, it should be noted that a pH indicator with longer wavelength emission is beneficial for intracellular applications. The pK_a values of SNARF-F and SNARF-Cl were determined to be 7.38 and 7.90, respectively (Figs. S1 and S2). It has been pointed out that the relatively high pK_a value of SNARF (pK_a = 7.62) is problematic for measuring the cytosolic pH of most cell lines (pH ~ 6.8–7.4).[2,4,7,8] In this regard, SNARF-F is more suitable as a pH indicator for intracellular pH measurement. The c log *P* values of SNARF, SNARF-F, and SNARF-Cl were calculated to be 4.99, 5.69, and 7.29, respectively.[9] These findings indicate that SNARF-F and SNARF-Cl are more hydrophobic than SNARF, and thus, should be more cell permeable than SNARF. In order to verify this, the cellular uptake of the two SNARF derivatives was quantitatively evaluated by flow cytometry. As shown in Figure 3, we confirmed more effective cellular uptake of SNARF-F and SNARF-Cl than of SNARF. Further, the localization of SNARF-F and SNARF-Cl were confirmed by a multistaining procedure

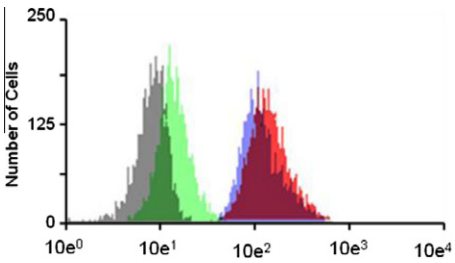

Figure 3. Flow cytometry analysis of the cellular uptake of the fluorescence of SNARF into V79 cells. The cells were incubated with SNARF-F (red), SNARF-Cl (blue) or SNARF (green) or without SNARF derivatives (gray).

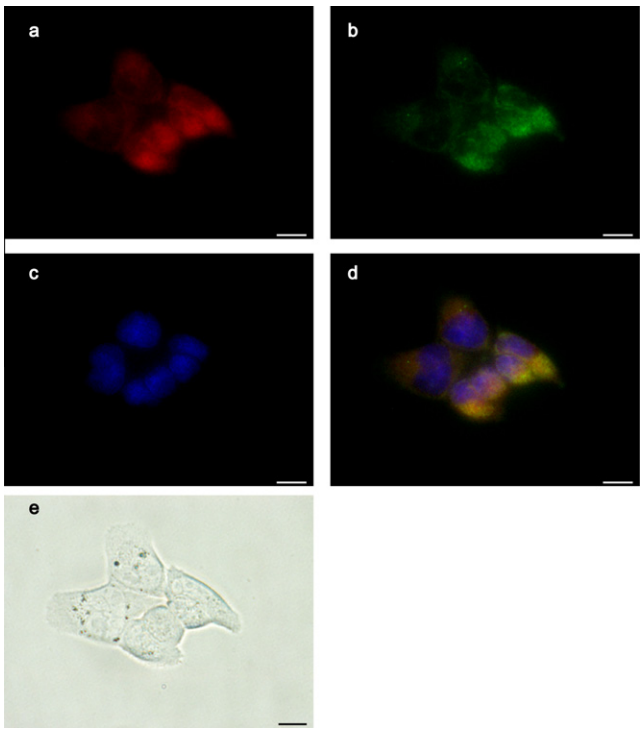

Figure 4. Microscopic images of V79 cells fluorescently labeled with SNARF-F, Mito Tracker Green FM and Hoechst 33258. Cells were imaged simultaneously for (a) SNARF-F, (b) Mito Tracker Green FM and (c) Hoechst 33258. The overlay image of (a), (b) and (c) is shown in (d). (e) is the transmission image. The scale bars (20 μm) are shown in the photograph.

(Fig. 4 and S3). Along with SNARF, SNARF-F and SNARF-Cl were localized mainly in the mitochondria and cytosol.[2,6a]

In conclusion, we designed and synthesized two new SNARF derivatives, SNARF-F and SNARF-Cl. Their photophysical and characteristic properties indicated both to be useful as dual-emission pH indicators. SNARF-F, in particular, appears to be more promising for intracellular application as compared to traditional SNARF, because SNARF-F combines the typical red shift of absorption and fluorescence with a lower pK_a together with higher cell permeability.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.105.

References and notes

1. (a) Willmann, J. K.; Bruggen, N. V.; Dinkelbuorg, L. M.; Gambhir, S. S. *Nat. Rev. Drug Disc.* **2008**, *7*, 591; (b) Kobayashi, H.; Ogawa, M.; Alford, R.; Choyke, P. L.; Urano, Y. *Chem. Rev.* **2010**, *110*, 2620.
2. Han, J.; Burgess, K. *Chem. Rev.* **2010**, *110*, 2709.
3. Whitaker, J. E.; Haugland, R. P.; Prendergast, F. G. *Anal. Biochem.* **1991**, *194*, 330.

4. Liu, J.; Diwu, Z.; Leung, W.-Y. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2903.
5. Nakata, E.; Yukimachi, Y.; Nazumi, Y.; Uto, Y.; Hashimoto, T.; Okamoto, Y.; Hori, H. *Chem. Lett.* **2010**, *39*, 734.
6. (a) Nakata, E.; Yukimachi, Y.; Nazumi, Y.; Uto, Y.; Maezawa, H.; Hashimoto, T.; Okamoto, Y.; Hori, H. *Chem. Commun.* **2010**, 3526; (b) Nakata, E.; Yukimachi, Y.; Kariyazono, H.; Im, S.; Abe, C.; Uto, Y.; Maezawa, H.; Hashimoto, T.; Okamoto, Y.; Hori, H. *Bioorg. Med. Chem.* **2009**, *17*, 6952.
7. The Molecular Probes Handbook 11th ed., Invitrogen Corp., 2010, Chapter 20, http://probes.invitrogen.com.
8. (a) Casey, J. R.; Grinstein, S.; Orlowski, J. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 50; (b) Swietach, P.; Vaughan-Jones, R. D.; Harris, A. L. *Cancer Metastasis Rev.* **2007**, *26*, 299.
9. C log *P* were estimated using PROLOGP 5.1 (log *P* Prediction Module of Pallas 3.0, CompuFrug International Inc., CA, USA).