http://dx.doi.org/10.5012/bkcs.2013.34.1.287

Reactive Cyanine Fluorescence Dyes Indicating pH Perturbation of Biomolecules

Jin Woo Park,[†,‡] Dong Jin Kim,[†] Kee-Jung Lee,[‡,*] and YoungSoo Kim[†,*]

[†]*Center for Neuro-Medicine, Brain Science Institute, Korea Institute of Science and Technology, Seoul 136-791, Korea*
[*]*E-mail: yskim@bio.kist.re.kr*
[‡]*Department of Chemical Engineering, Hanyang University, Seoul 133-791, Korea.* [*]*E-mail: leekj@hanyang.ac.kr*
Received September 19, 2012, Accepted October 22, 2012

Key Words : Fluorescence dyes, pH Sensitive dyes, Cyanine, Vinylsulfone group, Dye-labeled biomolecules

Maintaining the proper cellular pH is an essential condition for a biological system, and both intra- and extracellular pH levels are influenced by various pathophysiological conditions.[1] In normal physiological condition, extracellular pH value is maintained within a narrow range (pH 7.35-7.45).[2] Abnormally acidic environments are related to solid tumors, cystic fibrosis, asthma and a variety of renal conditions.[3] Thus, quantitative analysis of local pH using pH-sensitive probes is a promising tool for the analysis or diagnosis of biological events.[4] Optical imaging methods can provide noninvasive, real-time and high-resolution information about a wide range of biological targets.[5] As a result, many pH-sensitive fluorescent dyes have been developed as probes to allow visual inspection of pH in living cells, and some are commercially available.[6]

Cyanine dyes are advantageous for the design of multicolor probes due to the potential to alter absorbance/emission bands through variation of the number of methine carbons.[7] When the indole nitrogen atom is protonated, the resulting cation exhibits long wavelength absorption and a strong fluorescence band (Fig. 1(a)). The cation will also be deprotonated in less acidic media, resulting in a neutral nitrogen atom that gives rise to a blue-shifted absorption band and reduced fluorescence emission.[8-10] The majority of currently utilized pH indicating dyes are analogs of well-known cyanine, which may contain an *N*-hydroxysuccini-

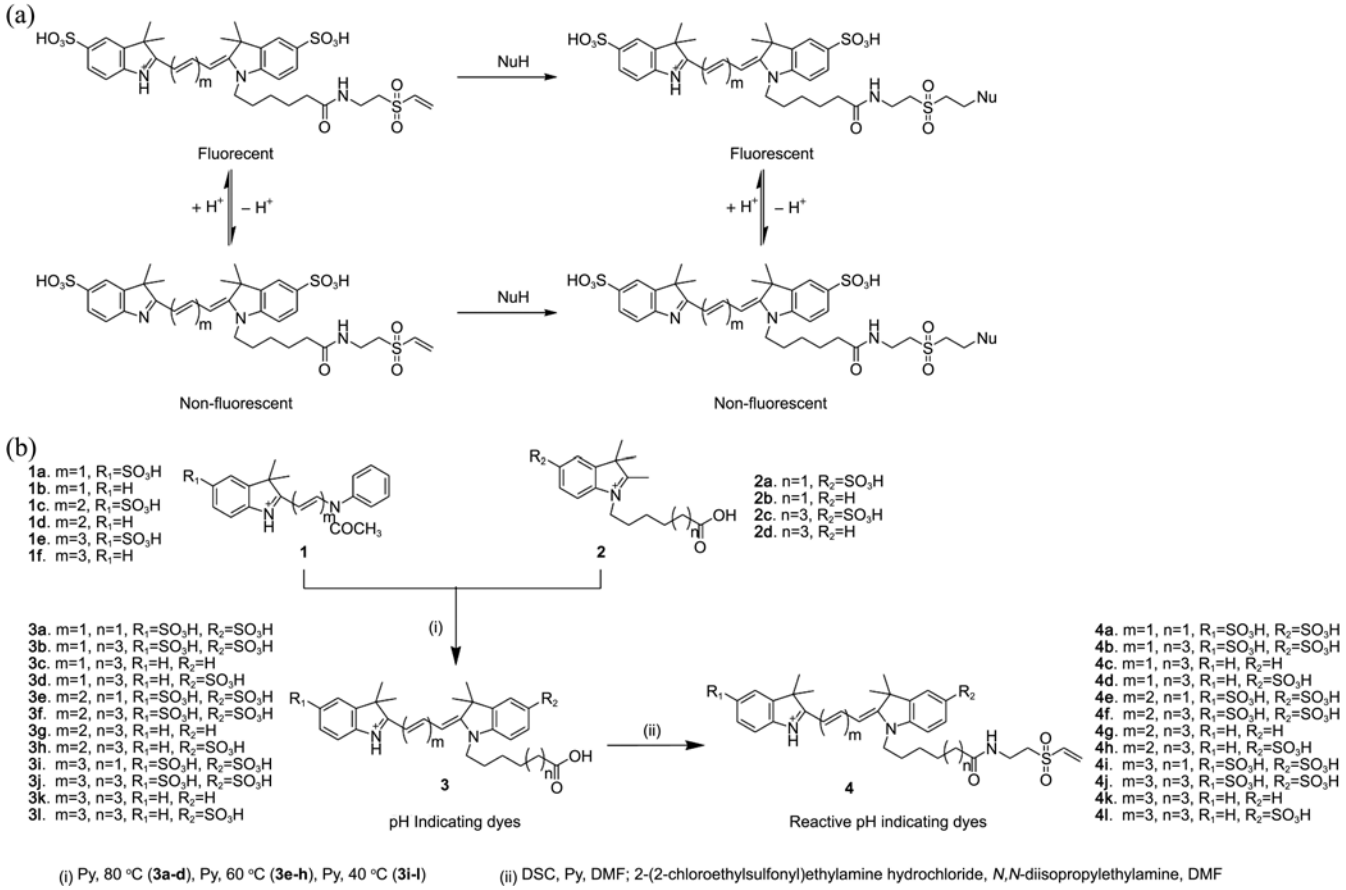


Figure 1. (a) Dyeing mechanism for VS cyanine dye with the nucleophiles (m = 1, 2, 3; Nu: nucleophiles) and optical characteristics of non-*N*-alkylated VS cyanine dyes; (b) Synthetic scheme for VS cyanine dyes.

mide ester (NHS) linkage or not.[11] The major concern associated with the cyanine-NHS system is its stability; namely, NHS is hydrolyzed rapidly in aqueous solution, especially under basic conditions.[6,7] In an effort to replace the labile NHS linkage, we recently reported the synthesis of a cyanine dye equipped with a highly reactive yet water stable vinylsulfone (VS) linkage.[12] Use of the VS modification is an attractive strategy due to its stability in aqueous media and high reactivity toward nucleophilic groups such as amines or thiols.[12-14]

We expected that variations in the electronic environment of the indole moiety would give rise to a novel cyanine dye that would exhibit dual absorbance spectra with respect to its pH environment. We first classified the VS cyanine dyes into 3 groups (m = 1, 2, and 3) and varied the number of sulfonic acid moieties (0, 1 and 2) and the length of alkyl chain (n = 1 and 3) between the dye and the VS group. In total, 12 compounds (**4a**-**4l**) were synthesized, as shown in Figure 1(b). Their absorbance and emission intensities were initially measured using a plate reader in 0.1 M sodium phosphate buffer media over a wide range of pH values (pH 2-11). The absorbance spectra of all samples were measured in various pH ranges, and their absorbance intensities were compared. The excitation wavelengths and intensities were set as 550 nm (m = 1), 650 nm (m = 2) and 750 nm (m = 3). When m = 1, all four compounds (**4a**-**4d**) exhibited a maximum absorbance value at 550 nm in acidic conditions, a new band (474 nm) began to appear around pH 7.4, and the peak at 550 nm nearly disappeared at pH values above 9.0. The observed color of the dyes also changed from red to orange as the pH values increased. Other compounds, specifically **4e**-**4h** (m = 2) and **4i**-**4l** (m = 3), also developed broad blue-shifted absorbance bands (501 nm, m = 2; 519 nm, m = 3) in basic media. The observed color of compounds **4e** and **4f** changed from blue to a dark red with increased pH value, and the color of compounds **4i** and **4j** also changed from green to a dark red (Supporting information, Fig. 1).

It was determined from the initial screening that hydrophilic compounds with m = 1 or 2 (**4a**, **4b**, **4e**, and **4f**) had similar optical properties, and their emission intensities were clearly distinguishable between pH 2-5 and 5-11 at the given excitation wavelength. Hydrophobic dyes (**4c**, **4d**, **4g**, and **4h**) were reserved for future research on whether organic media could address their limited aqueous solubility and were not pursued further. Near-infrared dyes (**4i**-**4l**) were also not studied further due to their low emission intensities. Among the hydrophilic compounds, **4b** and **4f** were selected as pH-indicating dye candidates due to their fast reaction rates. These compounds were studied for their binding to biomolecules and the optical properties of the resulting complexes.

The emission spectra of compounds **4b** and **4f** were compared with the commercially available pH-independent dyes FPR-552 and FPR-648, respectively.[12] Compounds **4b** and FPR-552 had the same maximum excitation wavelength (550 nm) and emission band (570 nm). However, the emission intensity of FPR-552 decreased to less than 10% of its maximum at pH 9.0, whereas the emission intensity of **4b** declined to 78% of maximum at pH 7.4 and to 9% at pH 9.0. Compounds **4f** and FPR-648 also had the same optical properties at pH 5.0. The emission intensity of FPR-648

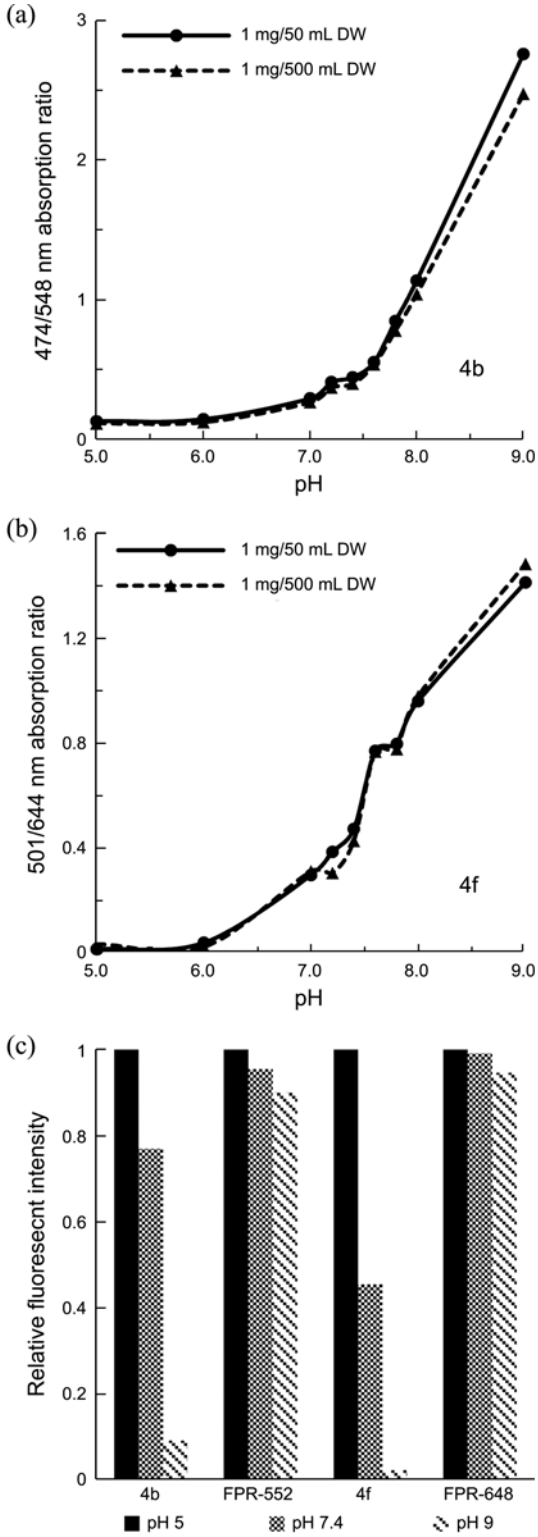


Figure 2. The ratios of absorption intensity of the blue shifted-absorbance bands and the original absorbance bands of **4b** (a) and **4f** (b); (c) Emission intensity comparison of pH insensitive cyanine dyes (FPR-552, FPR-648) and (**4b**, **4f**).

remained above 90% of maximum at pH 9.0, but the intensity of **4f** decreased to 42% of maximum at pH 7.4 and to 8% at pH 9.0. The main difference between **4b** and **4f** was the reduced emission intensity under the weakly basic conditions at pH 7.4. Under these conditions, **4b** did not exhibit a significant change in fluorescence, while the fluorescence of **4f** was less than half of the standard value (Fig. 2(c), Supporting information, Fig. 2). The essential requirement for a pH indicating dye is its response to subtle pH value variations in the physiologically and biologically significant pH region. In this respect, compound **4b** is not an optimal candidate because its emission intensity changed significantly above pH 7.4. However, the emission intensity of **4f** varied drastically between pH 5.0 and 8.0, which is applicable in biological systems.

The ratios of the blue shifted absorbance bands of compounds **4b** and **4f** with their absorbance intensities at pH 5.0 are shown at two different concentrations in Figure 2(a) and 2(b). The slope for compound **4b** was constant between pH 5.0 and 6.0, it increased linearly but slowly between pH 6.0 and 7.0, and it increased sharply above pH 7.5. However, the graph for **4f** exhibits a good linear slope between pH 6.0 and 9.0 that proves the utility of this dye for the detection of pH variation in that region. The main goal of this research was to find a dye candidate that possesses pH-responsive on/off emission properties; however, the absorbance ratio of the dye is also useful for confirming the measured pH value. After consideration of the optical properties of both candidates, **4f** was selected as the pH-indicator dye for study and used in the next step.

Having been chosen through the previous experiments, pH-indicating dye compound **4f** was used to label polymer nanoparticles, as well as biomolecules such as peptides and proteins. We wanted to see whether the fluorescence of the labeled protein would be diminished at neutral pH, as was observed for the dye compound alone. The Apopep-1 peptide (sequence: CQRPPR),[15] which is known to target apoptotic cells, was labeled with **4f** dye at the *N*-terminal amine of cysteine to afford **4f**-Apopep-1. Bovine Serum Albumin (BSA) was labeled with 1 and 5 molar equivalents of **4f** dye using the labeling protocol of vinylsulfone dyes to produce **4f**-BSA. Compound **4f** was also introduced to hydrophobically modified glycol chitosan (HGC) polymer nanoparticles,[16] which are known to accumulate readily in tumor tissues. To examine the change in fluorescence intensity with varying dye loadings, the HGC nanoparticles were labeled with 1, 10, and 100 molar equivalents of **4f** dye using the labeling protocol for Cy5.5, as described in the literature. All **4f**-labeled biomolecules and polymer nanoparticles were dissolved in sodium phosphate buffers of pH 5.0, 7.4, and 9.0 and the fluorescence intensities were measured. As shown in Figures 3(a) and 3(b), the fluorescence intensities at pH 7.4 decreased to less than 30% of those measured at pH 5.0. Fluorescence intensities at pH 9.0 were less than 10% of those observed at pH 5.0.

We have synthesized VS cyanine dyes that can be used to determine pH levels within a range of pH 5-9. Compound **4f** was selected due to its superior compatibility with biological research. Compound **4f** can be used to label protein molecules and maintains its optical properties when conjugated to a macromolecule, making it possible to observe pH distributions and changes in acidic and neutral pH conditions. It seems possible to use proteins labeled with **4f** as dyes exhibiting an ON/OFF feature because their fluorescent intensity was strongest at pH 5.0 and very weak at pH levels above pH 7.4. Apopep-1 peptide and HGC nanoparticles, which were used in this study, have been reported to bind to tumor cells and tissues. When conjugated with **4f**, the resulting pH-indicating probes may be utilized to examine the pH distribution within disease specific environments, determine non-specific binding sites, or contrast tumor tissues more brightly.

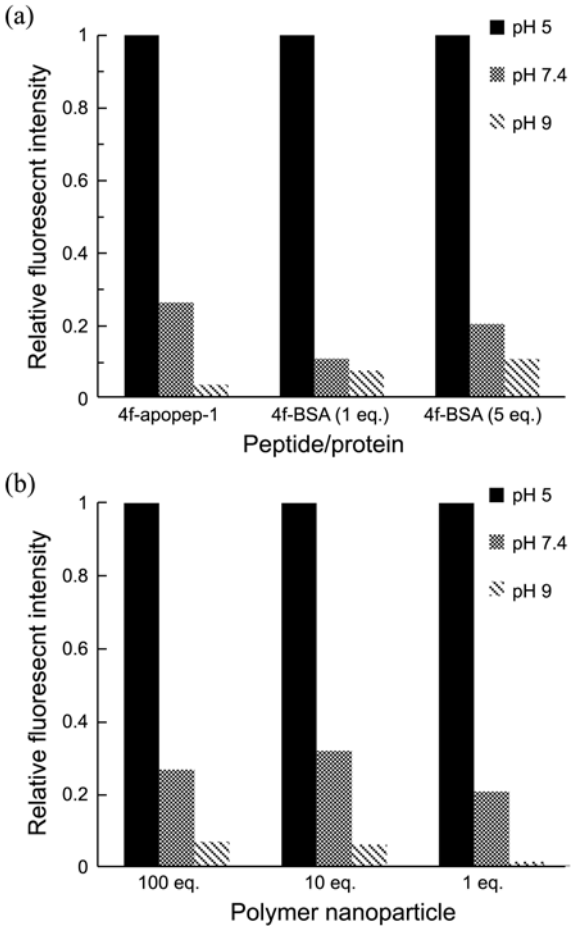


Figure 3. Comparison of fluorescence intensity at various pH levels for protein (a) and polymer nanoparticle (b) labeled with **4f**.

Experimental

Synthesis of compound **1a-1f**, **2a-d**, **3a-l** and **4a-l** was followed in the literatures (Supporting information).[7,12]

Apopep-1 peptide was synthesized through solid phase peptide synthesis (SPPS) by using the wang resin. The peptide was labeled in DMF with one molar equivalent of dye at the amine site of the *N*-terminal cysteine, while the

resin and the protecting group (NH_2-C(Trt)Q(Trt)R(Pbf)-PPR(Pbf)-resin) were still intact. After cleavage, the compound was refined by HPLC and freeze-dried to obtain fluorescent peptide **4f**-apopep-1. The fluorescent peptide was dissolved in sodium phosphate buffers of pH 5.0, 7.4, and 9.0, and the fluorescence intensity was measured.

Bovine serum albumin (BSA) was labeled with a dye by using the protocol made for vinylsulfone dyes. The amount of dye molecules to be used was determined to be 1 and 5 molar equivalent of the amount of protein. BSA was dissolved in 0.1 M sodium phosphate buffer (pH 9.5) to achieve 2 mg/mL concentration. Two separate tubes with BSA were prepared, and each was mixed with 1 and 5 molar equivalent of dye, which was dissolved in distilled water. The reaction was carried out in room temperature for 2 hours, and the final product **4f**-BSA (1 eq) and **4f**-BSA (5 eq) were obtained after separating unlabeled dye with PD-10 columns. The two compounds were diluted in sodium phosphate buffers of pH 5.0, 7.4, and 9.0, and their fluorescent intensity was measured.

The compound **4f** was introduced to hydrophobically modified glycol chitosan (HGC) polymer nanoparticles, which are known to easily accumulate in tumor tissues. The size of a HGC nanoparticle is approximately 300 nm, and contains many NH_2 groups that can be labeled with dye molecules. To examine the change in fluorescent intensity with varying amount of dye labeled, the HGC nanoparticles were labeled with 1, 10, and 100 molar equivalent amounts of dye molecules by using the labeling protocol for Cy5.5 indicated in the literature.[16]

Acknowledgments. This work was supported by the project No. 10033024 from the Advanced Technology Center (ATC) Support Program of Ministry of Knowledge Economy (Republic of Korea).

Supporting Information. Chemical and optical data of VS cyanine dyes (**4a-4l**); Labeling and refining method for **4f**-apopep-1, **4f**-BSA, and **4f**-HGC nanoparticle. This material is available free of charge *via* the Internet at http://pubs. acs.org.

References

1. Myochin, T.; Kiyose, K.; Hanaoka, K.; Kojima, H.; Terai, T.; Nagano, T. *J. Am. Chem. Soc.* **2011**, *133*, 3401.
2. Tang, B.; Yu, F.; Li, P.; Tong, L.; Duan, X.; Xie, T.; Wang, X. *J. Am. Chem. Soc.* **2009**, *131*, 3016.
3. Montcourrier, P.; Mangeat, P. H.; Valembois, C.; Salazar, G.; Sahuquet, A.; Duperray, C.; Rochefort, H. *Journal of Cell Science* **1994**, *107*, 2381.
4. Song, Y.; Salinas, D.; Nielson, D. W.; Verkman, A. S. *Am. J. Physiol. Cell Physiol.* **2006**, *290*, C741.
5. Ricciardolo, F. L.; Gaston, B.; Hunt, J. *J. Allergy Clin. Immunol* **2004**, *113*, 610.
6. Hilderbrand, S. A.; Weissleder, R. *Chem. Commun. (Camb)* **2007**, 2747.
7. Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S. *Bioconjug. Chem.* **1993**, *4*, 105.
8. Lavis, L. D.; Raines, R. T. *ACS Chem. Biol.* **2008**, *3*, 142.
9. Luo, S.; Zhang, E.; Su, Y.; Cheng, T.; Shi, C. *Biomaterials* **2011**, *32*, 7127.
10. Han, J.; Burgess, K. *Chem. Rev.* **2010**, *110*, 2709.
11. Zhang, Z.; Achilefu, S. *Chem. Commun. (Camb)* **2005**, 5887.
12. Park, J. W.; Kim, Y.; Lee, K. J.; Kim, D. J. *Bioconjug. Chem.* **2012**, *23*, 350.
13. Morales-Sanfrutos, J.; Lopez-Jaramillo, J.; Ortega-Munoz, M.; Megia-Fernandez, A.; Perez-Balderas, F.; Hernandez-Mateo, F.; Santoyo-Gonzalez, F. *Org. Biomol. Chem.* **2010**, *8*, 667.
14. Morales-Sanfrutos, J.; Lopez-Jaramillo, F. J.; Hernandez-Mateo, F.; Santoyo-Gonzalez, F. *J. Org. Chem.* **2010**, *75*, 4039.
15. Wang, K.; Purushotham, S.; Lee, J. Y.; Na, M. H.; Park, H.; Oh, S. J.; Park, R. W.; Park, J. Y.; Lee, E.; Cho, B. C.; Song, M. N.; Baek, M. C.; Kwak, W.; Yoo, J.; Hoffman, A. S.; Oh, Y. K.; Kim, I. S.; Lee, B. H. *J. Control. Release* **2010**, *148*, 283.
16. Nam, H. Y.; Kwon, S. M.; Chung, H.; Lee, S. Y.; Kwon, S. H.; Jeon, H.; Kim, Y.; Park, J. H.; Kim, J.; Her, S.; Oh, Y. K.; Kwon, I. C.; Kim, K.; Jeong, S. Y. *J. Control. Release* **2009**, *135*, 259.