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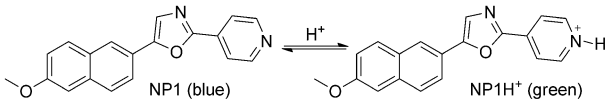
Fluorescent Probes

Measurement of pH Values in Human Tissues by Two-Photon Microscopy**

*Hee Jung Park, Chang Su Lim, Eun Sun Kim, Ji Hee Han, Tae Hee Lee, Hoon Jai Chun, and Bong Rae Cho**

Gastroesophageal reflux disease (GERD) is a common gastrointestinal disorder that is associated with the pH value of the esophageal lumen. It is a chronic condition in which mucosal damage is caused by the reflux of acidic gastric contents into the esophagus.[1] Nearly two thirds of the patients that have GERD-related symptoms are endoscopy-negative, which is called nonerosive reflux disease (NERD).[2,3] Currently available methods for the diagnosis of NERD include 24 h esophageal pH monitoring, endoscopic exam, and a proton-pump inhibitor (PPI) test.[4,5] However, none of these methods has yet been accepted as the gold standard. An interesting hypothesis on the pathophysiology of GERD proposes that as the dilated intercellular spaces (DIS) are widened and the tight junctions between the cells are weakend, the refluxed acid infiltrates into the esophagus, thereby stimulating the nociceptor.[6] To test this hypothesis and possibly diagnose NERD, it is crucial to measure the pH deep inside the esophageal tissue. However, except for a one-photon (OP), near-infrared (NIR), fluorescence-lifetime, pH-sensitive probe that has been applied to the mouse model,[7] there is no reliable method of measuring pH values deep inside human tissue.

An attractive approach to the determination of pH values deep inside living tissue is the use of two-photon microscopy (TPM). TPM, which employs two NIR photons as the excitation source, offers a number of advantages over one-photon microscopy (OPM), which include increased depth of penetration (more than 500 μm), localized excitation, and prolonged observation time as a result of the use of less-damaging, lower-energy light.[8] However, there has been no report on the targeted design and evaluation of a ratiometric pH probe that is applicable for measuring pH values by TPM. In this context, we have developed a ratiometric pH probe that is derived from 2-methoxy-6-(5-oxazolyl)naphthalene as the fluorophore and pyridine as the protonation site, with the expectation that protonation at the pyridyl nitrogen atom would result in a red-shifted fluorescence, thereby detecting the pH by ratiometry (Scheme 1). We adopted a naphthalene derivative as the fluorophore reporter because two-photon (TP) probes derived from similar compounds have been successfully utilized in live-tissue imaging.[9] Pyridine was chosen as the protonation site because the pK_a of pyridine is approximately 5.0, which is suitable to detect a slightly acidic environment. Herein, we report that NP1 is an efficient TP probe that can estimate pH values in live cells and human tissues through the use of TPM.



Scheme 1. Proposed mechanism of the equilibrium between NP1 and $NP1H^+$.

The synthesis of NP1 is summarized in the Supporting Information. Its solubility as determined by a fluorescence method is 5 μM in universal buffer solution (pH 7.0), which is sufficient to stain the cells (Figure S1 in the Supporting Information). Under this simulated physiological condition, NP1 has an absorption maximum at $\lambda_{abs} = 342$ nm ($\varepsilon = 17000\ M^{-1}cm^{-1}$) and a fluorescence maximum at $\lambda_{fl} = 500$ nm ($\Phi = 0.084$), which is almost 160 nm red-shifted from the λ_{abs}. The unusually large Stokes' shift that is observed for NP1 is likely to be a result of a great stabilization of charge-transfer excited state.

The absorption and emission spectra of NP1 showed gradual bathochromic shifts with the solvent polarity (E_T^N)[10] in the order, 1,4-dioxane < dimethylformamide (DMF) < EtOH < H_2O (Figure S2 and Table S1). The large bathochromic shift for the emission spectra (ca. 70 nm) indicates the utility of NP1 as a polarity probe. Furthermore, the value of λ_{fl} for NP1 in DMF is similar to that measured in HeLa cells (see Figure 2b below, see also Table S1), which suggests that this solvent can adequately represent the polarity of the intracellular environment.

The UV absorption of NP1 gradually decreased at 350 nm and concomitantly increased at 410 nm with a decrease in the pH value (Figure S3a), presumably as a result of protonation at the pyridyl nitrogen atom, thereby enhancing the intramolecular charge transfer (ICT) and λ_{abs} (Scheme 1). The behavior of the emission spectra was similar, except that the longer wavelength band decreased slightly. The relative

[*] Dr. H. J. Park,[+] C. S. Lim,[+] J. H. Han, Prof. Dr. B. R. Cho
Department of Chemistry, Korea University
145, Anam-ro, Sungbuk-gu, Seoul, 136-713 (Korea)
E-mail: chobr@korea.ac.kr
Prof. Dr. E. S. Kim, Prof. Dr. H. J. Chun
School of Medicine, Korea University (Korea)
T. H. Lee
Department of Chemistry, Chungnam National University (Korea)
[+] These authors contributed equally to this work.

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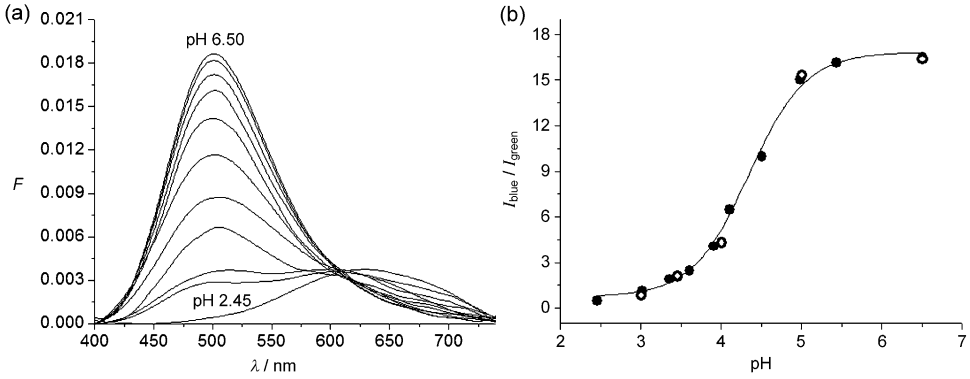

Figure 1. a) The change in the fluorescence spectra of NP1 (2.0 μM) with pH in universal buffer. b) Plots of I_{blue}/I_{green} versus pH as determined by one- (●) and two-photon mode (○) microscopy. Blue = 400–500 nm; green = 600–750 nm. The solid line is the theoretically fitted curve.

emission intensity ratios (I_{blue}/I_{green}) at 400–500 nm (I_{blue}) and 600–750 nm (I_{green}) increased by 12-fold as the pH value was changed from 2.45 to 6.50 (Figure 1a, see also Figure S3c). Moreover, the pK_a that was determined for NP1 (4.4) by the absorption and emission titration curves was identical, which demonstrates the reliability of these measurements (Figure 1b, see also Figure S3b). Furthermore, the plots of I_{blue}/I_{green} versus the pH value are linear at pH 3.5–5.0, which indicates that NP1 is suitable to determine pH values in this range. These results establish that NP1 can serve as a ratiometric probe that can estimate the pH value in the slightly acid region with reasonable accuracy.

We then evaluated the ability of NP1 to determine the pH value in a two-photon mode. The TP action cross-section ($\Phi\delta_{max}$) was determined by a fluorescence method with Rhodamine 6G as the reference.[11,12] The $\Phi\delta_{max}$ value of NP1 in DMF, which is a good model for the intracellular environment based on the similarity in the emission maxima (see above), is 155 GM at 740 nm (Figure S4). This value allowed us to obtain bright TPM images of the cells and human tissues that were labeled with NP1 (see below). Moreover, the I_{blue}/I_{green} ratio that was determined by the two-photon mode fitted well on the one-photon titration curve (Figure 1b, see also Figure S3c). These results confirm that NP1 can measure the pH value in a two-photon mode by ratiometry.

We next sought to utilize NP1 as a TP probe to determine the pH value in cellular environments. Using 740 nm TP excitation in scanning lambda mode, HeLa cells labeled with NP1 have a broad spectrum, which can be dissected into two Gaussian functions that have emission maxima at 450 nm (■, blue curve) and 575 nm (●, green curve), respectively (Figure 2b). The emission maxima are blue-shifted by 50 nm and 55 nm from those for NP1 and NP1H$^+$ measured in the buffer, respectively, which indicates that the probe environment in the cell is more hydrophobic than in the aqueous buffer (see above). Moreover, the shorter-wavelength band in the dissected Gaussian function decreased to the baseline at 575 nm (Figure 2b). However, the much smaller area of the green curve at 575–650 nm than that of the blue curve at 400–475 nm could cause errors in the measurement of I_{green} and I_{blue}/I_{green}. Because I_{green} can be doubled by increasing the detection window to 550–650 nm and the area of the blue curve at 550–575 nm is less than 10% of the blue curve, we collected the two-photon excitation fluorescence (TPEF) signals from the cell and tissue by using detection windows of 400–475 nm and 550–650 nm, respectively, to minimize these errors.

As the pH value in the lysosome is in the range of 4–5, there is a possibility that NP1 may accumulate in the lysosomes in the protonated form NP1H$^+$. To assess such

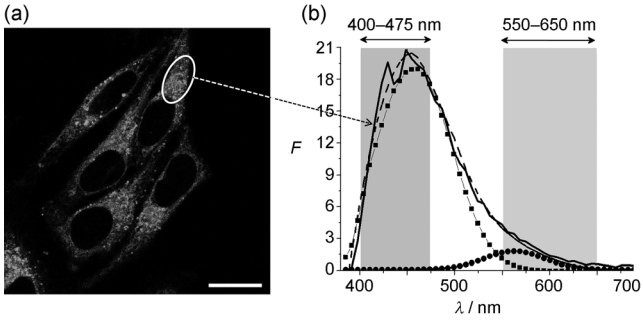

Figure 2. a) Pseudocolored TPM images of HeLa cells labeled with NP1 (5 μM). TPM image was acquired by using 740 nm excitation and fluorescent emission windows at 400–650 nm. Scale bar = 30 μm. Cells shown are representative images from replicate experiments ($n = 5$). b) TPEF spectrum acquired from the white-circled region in (a) (——). The spectrum was fitted to single Gaussian function (– – –), which was then dissected into two Gaussian functions with emission maxima at 450 (■) and 575 nm (●), respectively. Detection windows are 400–475 (dark gray) and 550–650 nm (bright gray)

a possibility, we performed a co-localization experiment with HeLa cells that were co-labeled with NP1 and Lysotracker Red (LTR), which is well-known, OP fluorescent probe for the lysosome.[13] The TPM image of NP1 showed partial overlap with the OPM image of LTR (Figure 3a–c, see also Figure S5). The Pearson's co-localization coefficient A, which describes the correlation of the intensity distribution between the channels,[14] was calculated by using Autoquant X2 software. The A value of NP1 with LTR was 0.23, whereas that of LTR with NP1 was 0.89, which indicates that the green domain in Figure 3a is a result of NP1 in the cytoplasm in addition to the NP1H$^+$ in the lysosomes.

A similar result was obtained in the co-localization experiment with NP1 and Lysotracker Blue (LTB, Figure 3d–h); the A value of NP1 with LTB was 0.19, while that of LTB with NP1 was 0.91 (Figure 3d and g). This outcome provides additional support for the above conclusion. Moreover, the TPM image that was collected at 550–650 nm overlapped well with the OPM image of LTB, with an A value of 0.86, which confirms that NP1H$^+$ exists predominantly in the lysosomes (Figure 3f–h). More importantly,

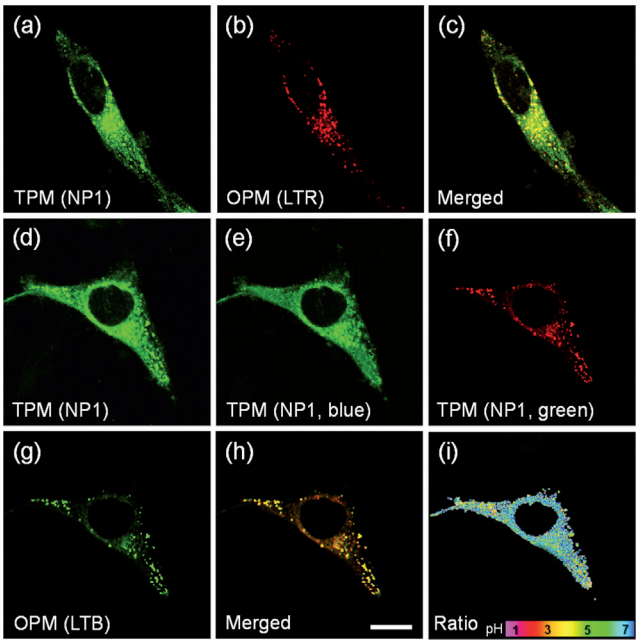

Figure 3. Pseudocolored TPM images of HeLa cells co-labeled with NP1 and LTR (a–c) or LTB (d–f) acquired by using 740 nm excitation and fluorescent emission windows at: a,d) 400–650 nm; e) 400–475 nm; f) 550–650 nm. b) OPM images acquired by using 543 nm and g) 365 nm excitation and fluorescent emission windows at 580–700 nm (b) and 390–450 nm (g). c,h) Merged images of (a) and (b), and (f) and (g), respectively. i) Ratio image constructed from e) and f). In all cases, the probe concentration was 5 μM. Scale bar = 20 μm. Cells shown are representative images from replicate experiments ($n = 5$).

NP1 is responsive to the pH value in the intracellular environment, showing acidic (orange spots) and neutral (blue domain) regions in the ratio image constructed from the blue and green windows (Figure 3i). NP1 has the additional benefits of high photostability as revealed by the negligible changes in the TPEF intensity in the NP1-labeled HeLa cells over 60 min (Figure S6) and small (10–20%) decrease in cell viability as measured by a Cell Counting Kit (CCK)-8 assay (Figure S7). These results establish that NP1 is capable of estimating the pH value in live cells with minimum interference from photostability and cytotoxicity issues.

We next sought to apply NP1 as a probe for measuring the pH value deep inside human tissues. We used stomach and esophagus tissues that were donated by esophagitis patients with esophageal mucosal injury as revealed by esophagogastroduodenoscope. The esophageal tissues were taken at the gastroesophageal junction (GEJ) and 5 cm above the proximal border of the lower esophageal sphincter (LES). Control tissues that were donated by control patients were also studied. As it takes a long time to stain the tissues, during which they may be damaged, an excess amount (20 μM) of NP1 was used to facilitate staining.

The TPM ratiometric images of the tissue slices labeled with 20 μM NP1 were obtained from the two collection windows at the depths of 90–180 μm to visualize the overall pH distribution (Figures S8 and S9). The pH values were estimated from the I_{blue}/I_{green} ratios and the titration curve (see above). In each site, a total of 70 I_{blue}/I_{green} ratios were acquired from 10 xy planes along the z-direction at the depths of 90–180 μm in seven tissue samples and used in the calculation. The results show that the pH value in the stomach is approximately 2.1 and is almost the same for the normal (2.1 ± 0.31) and esophagitis patients (2.1 ± 0.39) (Figure 4c). On the other hand, the pH value in the esophagus is significantly lower for esophagitis patients than in control patients, with pH values of (3.1 ± 0.41) and (4.7 ± 0.47) for the esophagitis patients versus (5.1 ± 0.25) and (7.2 ± 0.33) for the control patients, at the GEJ and 5 cm above the proximal border of the LES, respectively (Figure 4c). This outcome indicates that the esophagus of esophagitis patients is acidified by the acidic gastric contents that is refluxed from the stomach. Moreover, the ratiometric TPM images that were obtained at different depths reveal that the textures change slightly without appreciable differences in the pH value (Figures S8 and S9). These findings demonstrate that NP1 is capable of estimating the pH value in human tissues at depths in the range of 90–180 μm by using TPM.

In conclusion, we have developed a new TP probe, NP1, that has a significant TP action cross-section, a marked blue-to-green emission color change in response to changes in

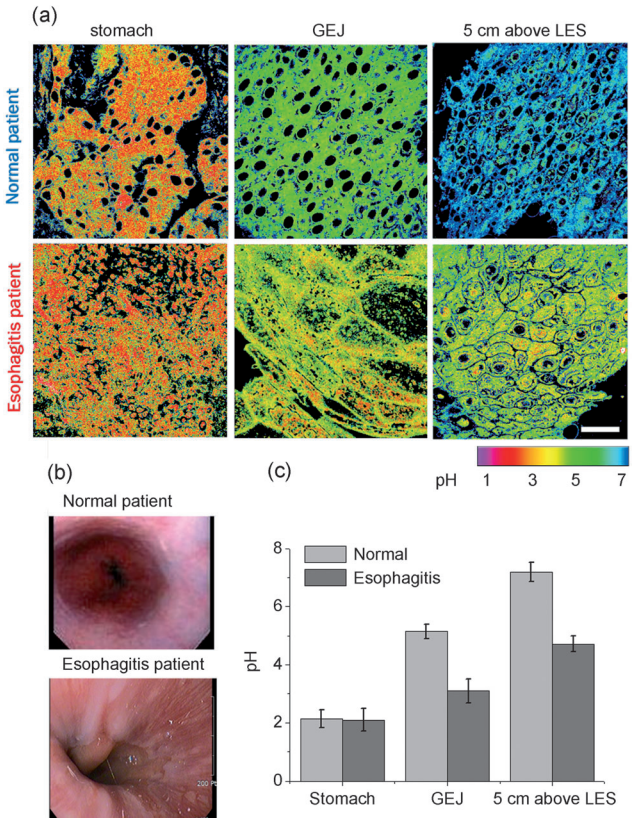

Figure 4. a) Ratiometric TPM images of stomach and esophagitis tissues labeled with 20 μM NP1 for 1 h. Images were acquired by using 740 nm excitation and fluorescent emission windows at: blue = 400–475 nm, green = 550–650 nm. The images shown are the representative images obtained at a depth of 120 μm ($n = 7$). Scale bar: 30 μm. b) Endoscope images of control and esophagitis patients. c) The average and standard deviation of the pH values that were estimated from the I_{blue}/I_{green} ratios and fluorescence titration curve.

pH value, high photostability, and low cytotoxicity. This new probe can estimate the pH value in live cells, as well as in human stomach and esophagus tissues at 90–180 μm depth by ratiometry, through the use of TPM. Therefore, this probe will find useful applications in measuring the pH value in various human tissues and possibly in the diagnosis of NERD.

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