Novel application of 4-nitro-7-(1-piperazinyl)-2,1,3-benzoxadiazole to visualize lysosomes in live cells

Kazuhiro Ishiguro¹, Takafumi Ando², and Hidemi Goto²

¹Molecular Biology and Pathogenesis of Gastroenterology, Nagoya University School of Medicine, and ²Department of Gastroenterology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, Aichi, Japan

BioTechniques 45:465-468 (October 2008)
doi 10.2144/000112912

4-Nitro-7-(1-piperazinyl)-2,1,3-benzoxadiazole (NBD-PZ) reacts with carboxylic acids in the presence of condensing agents and is utilized for the fluorescence detection of the generated derivatives in high performance liquid chromatography or capillary electrophoresis. Although the fluorescence intensity of derivatives of NBD-PZ–CH₂CH₂NH₂ with carboxylic acids is elevated at low pH, the pH-dependent fluorescence of NBD-PZ itself has not yet been investigated. In this study, we determined the fluorescence spectra of NBD-PZ at various pH and found that the fluorescence intensity of NBD-PZ was elevated dramatically at pH ≤7.0. When NBD-PZ was applied to live HGC-27 cells, CW-2 cells, IEC-18 cells, and RAW264.7 cells, we observed that NBD-PZ clearly visualized lysosomes under fluorescence microscopy within 3 min. These findings indicate a novel application of NBD-PZ to visualize lysosomes in live cells, a strategy that offers substantial cost savings over commercially available lysosomal probes.

Fluorescence agents containing nitrobenzoxadiazole (NBD) (excitation 470 nm, emission 540 nm) (Figure 1A) are used widely for sensitive detection of compounds in high performance liquid chromatography (1). For example, 4-nitro-7-(1-piperazinyl)-2,1,3-benzoxadiazole (NBD-PZ) (Figure 1B) was designed originally to tag carboxylic acids in the presence of activation agents such as diethyl phosphorocyanidate (2). Santa et al. have reported that acidic media (pH ≤3.0) elevate the fluorescence intensity of derivatives of NBD-PZ–CH₂CH₂NH₂ with carboxylic acids (3). They presumed that the fluorescence was quenched by an electron transfer from the nitrogen atom at the piperazine ring to the excited state of the fluorophore, and that this process—which is called the photo-induced electron transfer—was hampered in the acidic media, in which the nitrogen atom was protonated (3). Assuming this, photo-induced electron transfer would also occur in NBD-PZ and its fluorescence intensity should depend on pH. To evaluate the pH-dependent fluorescence of this compound, we prepared 10 mM NBD-PZ in dimethyl sulfoxide, diluted the solution to 10 μM with 0.05 M Britton-Robinson buffer at pH 9.0–2.0, and determined the fluorescence spectra of NBD-PZ with a fluorophotometer (RF-5000; Shimadzu, Kyoto, Japan). The fluorescence intensity of NBD-PZ was dramatically elevated at pH ≤7.0 (Figure 1C).

The lumenal environment of lysosomes is characterized by low pH via proton-pumping vacuolar ATPases (4). We therefore assessed whether NBD-PZ could be used to visualize lysosomes in live cells. We first evaluated the cytotoxicity of NBD-PZ using human gastric cancer-derived HGC-27 cells, human colon cancer-derived CW-2 cells, IEC-18 cells, and RAW264.7 cells, and observed that NBD-PZ clearly visualized lysosomes under fluorescence microscopy within 3 min. These findings indicate a novel application of NBD-PZ to visualize lysosomes in live cells, a strategy that offers substantial cost savings over commercially available lysosomal probes.
(RIKEN BRC Cell Bank, Ibaragi, Japan), rat intestinal epithelium–derived IEC-18 cells, and mouse macrophage–derived RAW264.7 cells (ATCC, Rockville, MD, USA). The cells (1 × 10^4) were seeded in 96-well plates. Twenty-four hours later, the cells were incubated with NBD-PZ (0–100 μM) for an additional 24 h. We determined cell viabilities with CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) using a luminometer (AB-2200-R; ATTO, Tokyo, Japan), and found that NBD-PZ did not affect the viabilities at 1 μM even after a 24-h incubation (Figure 1D). To see if NBD-PZ visualized lysosomes, the cells were incubated in culture medium containing 100 nM LysoTracker Red DND-99 probe (Molecular Probes, Eugene, OR, USA), for 30 min at 37°C. The medium was then replaced with one containing 1 μM NBD-PZ. After further incubation for 3 min at 37°C, the cells were viewed with a fluorescence microscope (BZ-8000; Keyence, Tokyo, Japan) using Texas Red and GFP-BP filters for LysoTracker Red DND-99 and NBD-PZ, respectively. NBD-PZ clearly visualized lysosomes in all cells tested (Figure 2A). Although lysosomes were visualized in the medium containing 1 μM NBD-PZ, the staining was preserved even after the cells were triple-washed with Dulbecco’s phosphate buffer (data not shown). Basic amines are known to accumulate in cellular compartments with low internal pH (5). On the other hand, the visualization of lysosomes was hampered in the cells pretreated with bafilomycin A_1, a vacuolar ATPase inhibitor that increases lysosomal pH (6) (Figure 2B). These findings suggest that NBD-PZ stains lysosomes in live cells via its pH-dependent fluorescence and accumulation in lysosomes.

Lysosomes are membrane-bound organelles and are involved in the degradation of macromolecules and pathogens in endocytosis, phagocytosis, and autophagy (4). Recent studies have indicated lysosomes as targets for cancer therapy via

### Table 1. Comparison of Cost

<table>
<thead>
<tr>
<th>Probe</th>
<th>Price (USD)</th>
<th>Size</th>
<th>Concentration</th>
<th>Volume</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBD-PZ</td>
<td>$60.05</td>
<td>400 μmol</td>
<td>1 μM</td>
<td>400 L</td>
<td>$0.15/L</td>
</tr>
<tr>
<td>LysoTracker</td>
<td>$207.00</td>
<td>1 μmol</td>
<td>100 nM</td>
<td>10 L</td>
<td>$20.70/L</td>
</tr>
<tr>
<td>LysoSensor</td>
<td>$201.00</td>
<td>1 μmol</td>
<td>100 nM</td>
<td>10 L</td>
<td>$20.10/L</td>
</tr>
</tbody>
</table>

Prices are per 1 L of working solution. LysoTracker (Blue, Green, and Red) and LysoSensor (Blue and Green) are lysosomal probes obtained from Molecular Probes.

*Working concentration for the similar visualization of lysosomes.

**Total volume of working solution.

![Figure 2. Visualization of lysosomes with NBD-PZ under fluorescence microscopy.](image)

(A) After incubation with 100 nM LysoTracker Red DND-99 for 30 min at 37°C, the cells were incubated with 1 μM NBD-PZ for 3 min and viewed with the fluorescence microscope. Bars, 10 μm. (B) RAW264.7 cells were incubated for 2 h at 37°C in the absence or presence of 25 nM bafilomycin A_1, and then with 1 μM NBD-PZ for 3 min.
death pathways involving lysosomal membrane permeabilization and the release of cathepsins into the cytosol (7,8). NBD-PZ is now available from Tokyo Chemical Industry (Tokyo, Japan or TCI AMERICA, Portland, OR, USA) and Sigma-Aldrich (St. Louis, MO, USA) for $60.05 and $108.70 USD for 100 mg (about 400 μmol), respectively. As shown in Table 1, NBD-PZ is much less expensive than other lysosomal probes. We expect that the novel application of NBD-PZ can facilitate the investigation of lysosomal dynamics in live cells. Furthermore, since the intracellular pH of tumor cells is considerably more acidic than that of normal cells (9,10), the combined application of a fluorescence endoscope and NBD-PZ may potentially have in vivo imaging applications, such as improving the detection of intraepithelial neoplasias in the gastrointestinal tract.

ACKNOWLEDGMENTS

We thank Chie Moriyama for her technical assistance. This work was supported in part by grants from the Japan Foundation for Applied Enzymology and the Japan Society for the Promotion of Science.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES


Received 4 April 2008; accepted 11 June 2008.

Address correspondence to Kazuhiro Ishiguro, Molecular Biology and Pathogenesis of Gastroenterology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. e-mail: kio@med.nagoya-u.ac.jp

To purchase reprints of this article, contact: Reprints@BioTechniques.com