Original article

A 1,8-naphthalimide-derived turn-on fluorescent probe for imaging lysosomal nitric oxide in living cells

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ABSTRACT

Nitric oxide has played an important role in many physiological and pathological processes as a kind of important gas signal molecules. In this work, a new fluorescent probe LysoNO-Naph for detecting NO in lysosomes based on 1,8-naphthalimide was reported. LysoNO-Naph has sub-groups of o-phenylene-diamine as a NO reaction site and 4-(2-aminoethyl)-morpholine as a lysosome-targetable group. This probe exhibited good selectivity and high sensitivity (4.57 μmol/L) toward NO in a wide pH range from 4 to 12. Furthermore, LysoNO-Naph can be used for imaging NO in lysosomes in living cells.

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1. Introduction

Fluorescence imaging has unique advantages to study cellular functional molecules in vitro and/or in vivo [1–3]. By attaching a sub-cellular organelle specific group, fluorescent probes can be used to detect the localization and monitor the movement of target biomolecules [4–7]. Lysosomes are round-shaped organelles that contain acid hydrolase enzymes that break down waste materials and cellular debris. The interior of the lysosomes is acidic at pH 4.8 compared to the slightly basic cytosol at pH 7.2. Lysosomes were traditionally treated only as the cell recycling centers. They can make the senescent cellular debris and needless macromolecules into small molecules, and then the cells utilize the small molecules for living [8]. In recent years, lysosomes have been recognized as an more important organelle in cells. They are related to many diseases such as cancer, neurodegenerative disorders, and cardiovascular diseases. It is extremely urgent to make real-time detection and imagining of lysosomes as well as inside molecules. In recent years, fluorescent probes for staining lysosomes [9–11] or image lysosomal H₂S [12], NO, [13] HOCl [14] and pH [15] have been reported.

Nitric oxide (NO) as a uncharged free radical exists everywhere in space [16,17]. Because of its highly chemical reactivity, early studies recognized NO as a kind of poison without other biological significance. As time goes on, we started to realize that NO is an indispensable messenger molecule in the cardiac disorders, immune system disorder and neurodegeneration [18–20]. It makes white blood cells kill tumor cells and bacteria, and much like neurotransmitter, it can dilate blood vessels. Disclosing the distribution of NO and its relationship with bodily agents will be critical for the treatment for the disorders mentioned above. Some fluorescent probes, including o-phenylenediamine based fluorescent probes [21–29] and copper complex based compounds [30], have been reported to detect NO with some successful applications to image NO in living cells.

In this paper, we reported a new fluorescent probe LysoNO-Naph to detect NO in lysosomes. Derived from 4-bromo-1,8-naphthalic anhydride, the NO reactive site o-phenylenediamine and a lysosome target group 4-(2-aminoethyl)-morpholine were introduced to the fluorophore. This probe can be easily obtained with high yield. As shown in Scheme 1, LysoNO-Naph reacted with NO to form a triazole ring and simultaneously displayed a turn-on blue fluorescence. Its propreties of imaging lysosomal NO were also studied. The experimental details are given in Supporting information.

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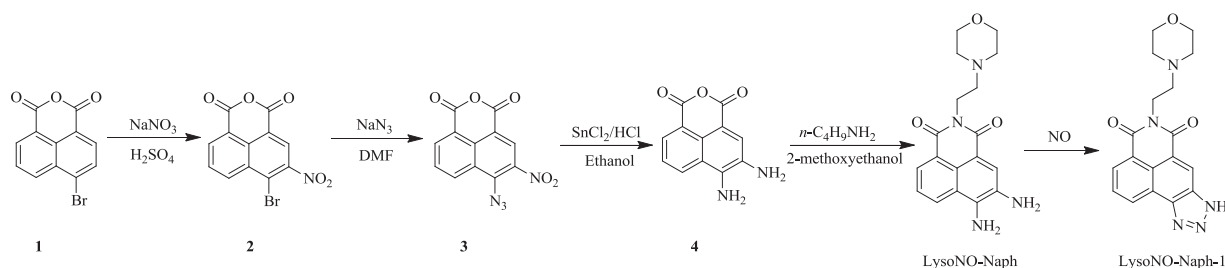
Scheme 1. Synthesis of LysoNO-Naph and the reaction process with NO.

2. Experimental

2.1. Materials and instruments

Unless otherwise noted, all reagents were obtained from Aldrich and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Burker 400 MHz spectrometer. UV-visible absorption spectra were collected on Agilent Cary 60 UV/VIS spectrophotometer. Fluorescence emission spectra were performed on Cary Eclipse Fluorescence Spectrophotometer (Serial No. FL0812-M018). Compounds **2–4** were synthesized with a modified method according to [31].

HPLC-MS analysis was performed on Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS using an HPLC system composed of a pump (Agilent ZORBAX Eclipse Plus C18 2.1 mm × 50 mm) and a LysoNO-Naph detector (254 nm).

2.2. Synthesis of compound 2–4

2.2.1. Synthesis of compound 2

NaNO$_3$ (2.0 g, 23.53 mmol) was carefully added to a suspension of 4-bromo-1,8-naphthalic anhydride (5.0 g, 18.1 mmol) in concentrated sulphuric acid (30 mL). The solution was stirred for 3 h at 0 °C, and then moved to room temperature for 1 h. The solution was poured into 300 mL of ice-water, filtered to give yellow solid. The crude product was recrystallized in glacial acetic acid to yield product as yellow solid (3.76 g, 65%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.93 (s, 1H), 8.84 (d, 1H, J = 8.6 Hz), 8.75 (d, 1H, J = 7.9 Hz), 8.23–8.16 (m, 1H). MS (EI) calcd. for C$_{12}$H$_4$BrNO$_5$ [M$^+$] 320.93, found 320.93.

2.2.2. Synthesis of compound 3

After a mixture of compound **2** (2 g, 6.32 mmol) and DMF (25 mL) was stirred at room temperature for 10 minutes, NaN$_3$ (0.608 g, 9.35 mmol) was carefully added, and the mixture was stirred for 3 h at room temperature. The solution was poured into 200 mL of ice-water, and the precipitate was filtered and washed with water. After drying, compound **3** was obtained (1.2 g, 68%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.69 (d, 1H, J = 8.5 Hz), 8.34 (d, 1H, J = 7.2 Hz), 8.08 (s, 1H), 7.69 (t, 1H, J = 7.9 Hz). MS (EI) calcd. for C$_{12}$H$_4$N$_4$O$_5$ [M$^+$] 284.02, found 284.02.

2.2.3. Synthesis of compound 4

After a mixture of SnCl$_2$ (3 g, 13.31 mmol) and concentrated hydrochloric acid (8 mL) was stirred at room temperature for 10 min, compound **3** was slowly added, and the mixture was stirred for 40 minutes at 50 °C. Then stirred at 80 °C with ethanol (8 mL) for another 3 h. After the reaction, mixture was cooled down to room temperature, the precipitate was filtered and washed with water. After drying, red compound **4** was obtained (0.54 g, 81‰). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.58 (d, 1H, J = 8.5 Hz), 8.21 (d, 1H, J = 7.1 Hz), 7.89 (s, 1H), 7.62–7.56 (m, 1H), 6.89 (s, 2H), 5.28 (s, 2H). MS (ESI) calcd. for C$_{12}$H$_9$N$_2$O$_3$ [M + H$^+$] 238.13, found 238.13.

2.3. Synthesis of LysoNO-Naph

To a solution of 3,4-diamino-1,8-naphthalic anhydride (200 mg, 0.88 mmol) in 20 mL 2-methoxyethanol was added 2.0 eq 4-(2-aminoethyl)-morpholine (231 μL, 1.76 mmol). The mixture was then heated at 125 °C for 5 h and monitored by TLC. After the completion of the reaction, the residue was purified by silica gel column (CH$_2$Cl$_2$:CH$_3$OH = 20:1) to give 150 mg LysoNO-Naph as red powder in 51% yield. M. p. 225–227 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.49 (d, 1H, J = 8.3 Hz), 8.20 (d, 1H, J = 7.0 Hz), 7.92 (s, 1H), 7.58–7.52 (t, 1H), 6.51 (s, 2H), 5.15 (s, 2H), 4.14 (t, 2H, J = 6.9 Hz), 3.56–3.51 (m, 4H), 2.53–2.50 (m, 2H), 2.45 (s, 4H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.50 (s), 163.57 (s), 136.88 (s), 131.00 (s), 128.47 (s), 127.70 (s), 124.19 (s), 123.75 (s), 122.11 (s), 121.31 (s), 120.06 (s), 108.76 (s), 66.68 (s), 56.32 (s), 53.89 (s), 36.82 (s). HRMS (ESI) calcd. for C$_{18}$H$_{21}$N$_4$O$_3$ [M + H$^+$] 341.1614, found 341.1610.

2.4. Culture of CHO cells and fluorescent imaging:

CHO cells was hatched in an atmosphere of 5% CO$_2$ and 95% air in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) at 37 °C. The cells were seeded in 24-well flat-bottomed plates and then incubated for 72 h at 37 °C under 5% CO$_2$. After adding LysoNO-Naph (5 μmol/L) to the cells, incubate them for another 30 min. Then use phosphate-buffered saline (PBS, 10 mmol/L) to wash cells for three times. Added 100 μmol/L of SNP (release NO, 50 μmol/L) and Neutral Red (2 μmol/L) to the cells, incubate them for 30 min. Fluorescence imaging was observed under a confocal microscopy (Olympus FV1000) with a 60× objective lens.

3. Results and discussion

As lysosomes have an acid interior with pH from 4.0 to 6.0, lysosome-targetable fluorescent probes must remain unaffected fluorescence without the interaction with analyte at least in this pH range. So, the pH effect on the absorption and fluorescence of LysoNO-Naph were firstly studied. As shown in Fig. 1, the maximum absorption peak of LysoNO-Naph at 462 nm remained stable in the pH range from pH 3.80 to 12.54. Accordingly, LysoNO-Naph did not fluoresce in this pH range. In much more acidic solutions, the maximal absorption peak moved to shorter wavelength. And in much more basic solutions, the maximal absorption peak reminded but with decreased fluorescence intensity. Hence, LysoNO-Naph is suitable to be applied to monitor lysosomal NO, since it is stable in pH from 4.0 to 6.0 without any changes. The polarity effects on absorption and fluorescence were also examined. As indicated in Figs. S2 and S3 (see Supporting information), LysoNO-Naph is a typical intramolecular charge-transfer (ICT) type of fluorescent dyes. With the increase of polarity, the absorption and emission wavelengths would be red-shifted.

The absorption and fluorescence spectra of LysoNO-Naph with the addition of NO were obtained in mixture solutions of
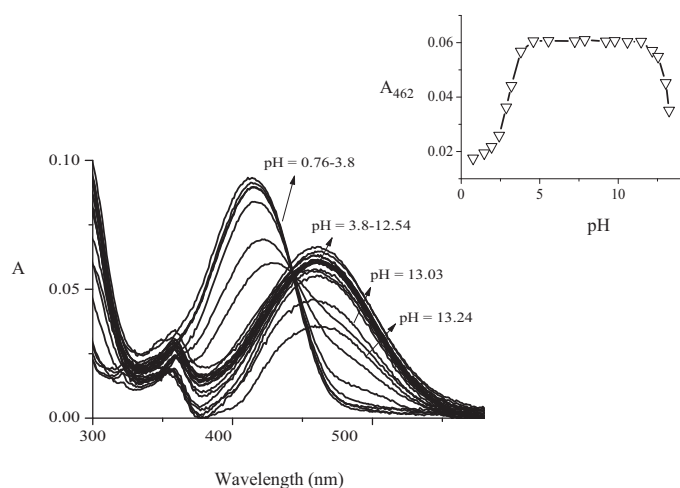
Fig. 1. Effects of pH on the absorption of LysoNO-Naph.

Fig. 3. (a) Fluorescence changes and (b) fluorescence ratio (F_{454}/F_0) changes of LysoNO-Naph (10 μmol/L) upon addition of SNP (0–1 eqiv.).

acetonitrile and PBS buffer s (1:1, v/v, pH 7.4, 20 mmol/L). LysoNO-Naph has a main absorption band at 462 nm and a second absorption band at 368 nm (Fig. 2a). When 25 equiv. of SNP (NO donor) were added to the solution of LysoNO-Naph, a significant decrease in the 462 nm absorption and a blue-shifted absorption band centered at 368 nm, which was attributed to the formation of compound LysoNO-Naph-1 and increased in intensity, were observed with an isoabsorption point at 405 nm. In the same time, the fluorescence emission band centered at 460 nm appeared and increased in intensity ($\Phi_F = 0.23$) (Fig. 2b). We found that if the concentration of added SNP was 25 equiv., the reaction can be finished within 20 min (insets in Fig. 2). With much more SNP like 50 equiv. (Fig. S1 in Supporting information), the reaction can be finished in a shorter time (10 min). Then we decided to use 25 equiv. of SNP to examine the performance of LysoNO-Naph in following experiments.

Fig. 3 displayed the titration experiments of LysoNO-Naph with the addition of 0–1 equiv. SNP. There was a good linearity between the ratio in the fluorescent intensity (F_{454}/F_0) and concentrations of SNP (in the range of 0–1 equiv.) with a detection limit to 4.57 μmol/L (Fig. 3). Moreover, the light blue fluorescence change can be easily recognized by naked eyes. The reaction product was analyzed by HPLC-MS. As shown in Fig. 4, the blue fluorescence product was proved to be LysoNO-Naph-1.

We also tested the selectivity of LysoNO-Naph with different kind of reactive species (ROS) and reactive nitrogen species (RNS)
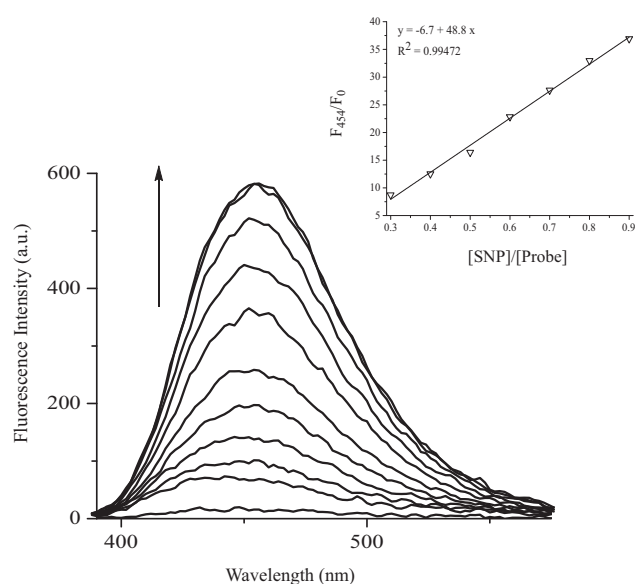
including NO, NO_2^-, NO_3^-, H_2O_2 and ClO^-. As shown in Fig. 5, when LysoNO-Naph was added with 25 equiv. SNP, a great fluorescent increase at 454 nm and a great absorption band at 368 nm were observed. With the addition of 100 equiv. of NO_2^-, NO_3^-, H_2O_2 and ClO^-, no obvious changes in fluorescence and absorption were observed. In this regard, LysoNO-Naph has a high selectivity for NO.

We next applied LysoNO-Naph for fluorescence imaging of NO in CHO cells (Chinese Hamster Ovary). After incubation with 5 μmol/L LysoNO-Naph in culture medium for 30 min, cells were washed with phosphate buffered saline (PBS, 10 mmol/L, pH 7.4) for three times to remove needless probe. There was a very weak fluorescence existed in confocal image (Fig. 6a). Then 50 μmol/L SNP was added and treated for another 30 min, and a strong blue fluorescence was observed, indicating the detection of cellular NO by LysoNO-Naph (Fig. 6b). In order to identify organelles from the fluorescence imaging of LysoNO-Naph in cells, we then carried out fluorescence localization by co-staining cells with commercially available organelle-specific dyes (Fig. 6c and d). The highly blue fluorescent regions with LysoNO-Naph (Fig. 6b) and signals from NR (Fig. 6c) overlapped perfectly (Fig. 6d), indicating that these
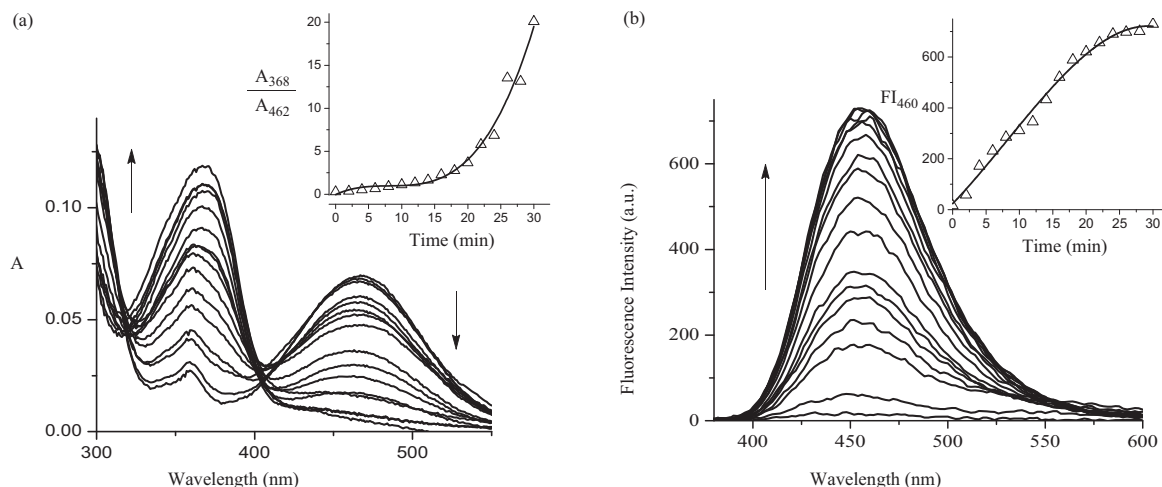

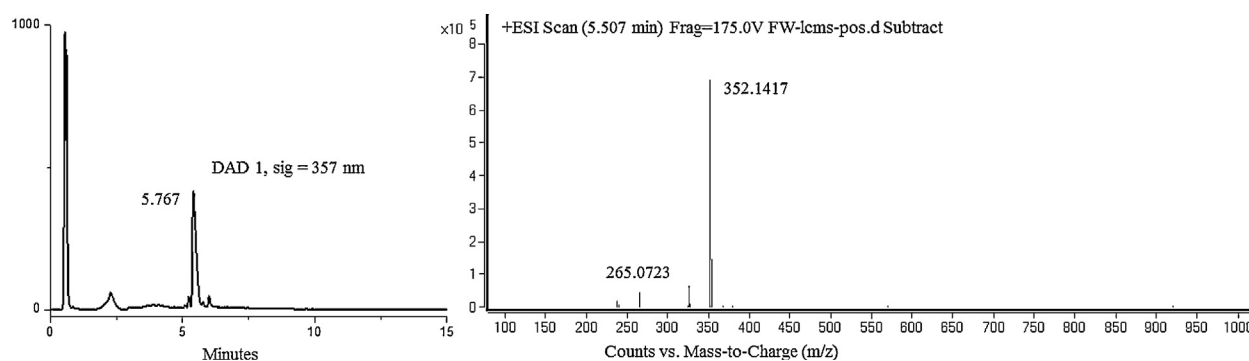**Fig. 2.** (a) Absorption and (b) fluorescence of LysoNO-Naph (10 μmol/L) with SNP (25 eqiv.) from 0 to 30 min.

Fig. 4. HPLC-MS of the reaction product of LysoNO-Naph (10 μmol/L) and SNP (25 eqiv.).

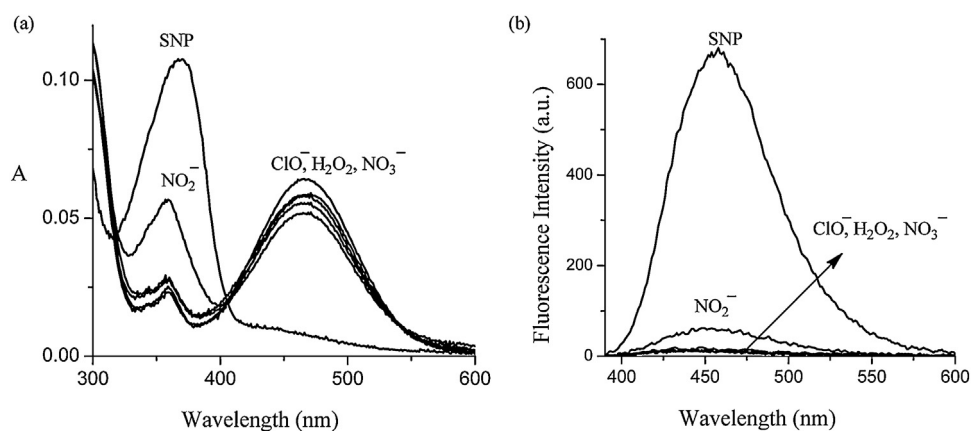**Fig. 5.** (a) Absorption and (b) fluorescence of LysoNO-Naph (10 μmol/L) with the addition of ClO⁻, H_2O_2, NO_3^-, NO_2^- (100 eqiv.) and SNP(25 eqiv.).

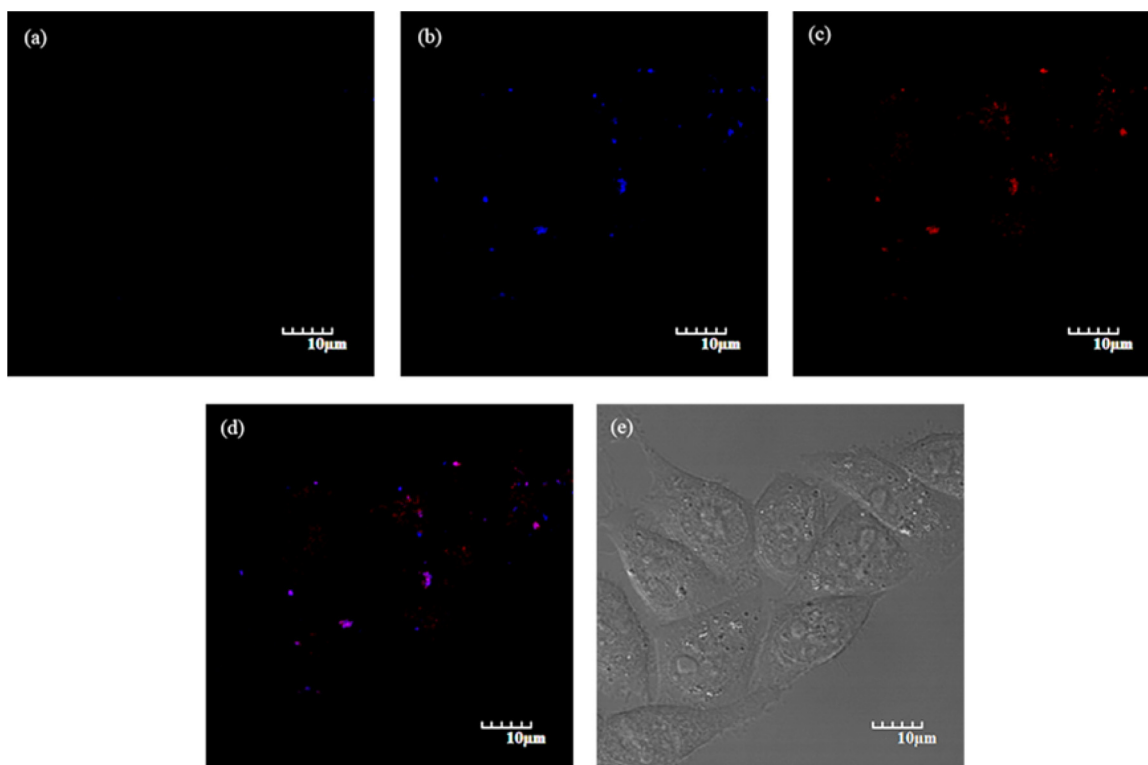**Fig. 6.** Fluorescence images of CHO cells incubated with 10 μmol/L LysoNO-Naph and NO. Cells treated with LysoNO-Naph (a) in the absence and (b) presence of 50 eqiv. SNP. (c) Image of lysosome tracker (Neutral Red). (d) Fluorescence co-localization imaging of LysoNO-Naph and Neutral Red. (e) Bright field image.

fluorescent regions were localized at lysosomes. These experiments demonstrated that LysoNO-Naph can be used as a fluorescent probe to detect NO in lysosomes of living cells.

4. Conclusion

In conclusion, we have reported a fluorescent probe LysoNO-Naph for imaging NO in lysosomes based on the 1,8-napthalimide. The probe can be obtained easily with a high yield. Its insensitivity to pH from 3.8–12 made it possible to be used for cellular lysosome imaging. LysoNO-Naph displayed a high selectivity for NO and high sensitivity with a detection limit down to 4.57 μmol/L.

Acknowledgment

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cclet.2016.06.016.

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