A Lysosome-Targetable and Two-Photon Fluorescent Probe for Monitoring Endogenous and Exogenous Nitric Oxide in Living Cells

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Ⓢ *Supporting Information*

ABSTRACT: A lysosome-specific and two-photon fluorescent probe, **Lyso-NINO**, demonstrates high selectivity and sensitivity toward NO, lower cytotoxicity, and perfect lysosomal localization. With the aid of **Lyso-NINO**, the first capture of NO within lysosomes of macrophage cells has been achieved using both two-photon fluorescence microscopy and flow cytometry.

Recently, it has been discovered that lysosomal functions are subtly regulated by nitric oxide (NO), a ubiquitous cellular messenger molecule in the cardiovascular, nervous, and immune systems.[1] NO exhibits complex effects on the catabolic autophagy process,[2] which involves the degradation of a cell's own components through the lysosomal machinery, to supply energy and nutrient sources for cell growth,[3] and is also closely related to various disorders and diseases including lysosomal storage disorders,[4] Gaucher's disease,[5] and Danon disease.[6] Endogenous NO, induced by some stimulants including Lipopolysaccharide (LPS) and Interferon-r (IFN-r), has been reported to react with several related proteins outside lysosomes to drive the autolysosome process by releasing the upstream signals.[7] However, the effects of NO on lysosomes are far from being completely understood. Owing to the dynamic transformation of the morphology and components of the highly heterogeneous lysosomes, it is difficult to find solid evidence of direct interactions between NO and components within lysosomes, but that does not mean they are non-existent. So far, scientists have no tool to determine whether NO enters into lysosomes, not to mention its quantification.

To our knowledge, fluorescent probes—powerful tools in cell biology—can hardly meet the requirements for monitoring lysosomal NO. A variety of NO-sensitive fluorescent molecular probes have already been developed to image intracellular NO under one-photon microscopy (OPM),[8] which has significantly enriched our knowledge about NO homeostasis and NO's critical roles in many biological processes.[9] Nevertheless, none of these probes is well suited for lysosomal applications; they all failed to specifically localize in lysosomes and monitor lysosomal NO.

Besides the lack of lysosomal specificity, another disadvantage of the known NO probes is the lower two-photon fluorescence activity, which gives them poor applicability in two-photon microscopy (TPM). The TPM technique, exciting the fluorophores with NIR laser pulses, has become a potent alternative to OPM because it overcomes latter's problems of shallow penetration depth, photodamage, and cellular auto-fluorescence.[10]

Herein, based on the principle of photoinduced electron transfer (PET), a lysosome-targeted and two-photon NO fluorescent probe, **Lyso-NINO**, has been designed through integration of NO-capturing *o*-phenylenediamine, lysosome-targeting (aminoethyl)morpholine, and efficient two-photon fluorophore naphthalimide, as shown in Scheme 1. *o*-Phenyl-

Scheme 1. Chemical Structure of Lyso-NINO and the Reaction of Lyso-NINO with NO

enediamine, employed not only as an electron donor (a fluorescence quencher) for naphthalimide but also as a NO trapper, was directly tethered to the imide position of naphthalimide. Accordingly, the 4 position of naphthalimide could be preserved and further modified with 4-(2-aminoethyl)-morpholine. The alkylmorpholine group, possessing a lower pK_a value, targets lysosomes that contain a lot of acid hydrolases in the pH range from 4.5 to 5.5.[11,12]

First, our study on **Lyso-NINO** and **Lyso-NINO-T**'s pH responses confirms the probe's suitability to lysosomal pH range. As shown in Figure 1, the pK_a of **Lyso-NINO-T** is 5.61 ± 0.02, which is in good accord with the pK_a value of the well-known Lysosensor DND-189 (pK_a = 5.80).[12] This should be attributed to the lower pH response of the morpholine group. Compared with **Lyso-NINO-T**, **Lyso-NINO** has a lower pK_a value, 3.47 ± 0.01, which is ascribed to the pH independence of *o*-phenylenediamine. From these results, we could predict that, in lysosomal pH ranges such as pH 4.5−5.5, there would be very weak fluorescence for **Lyso-NINO**, yet **Lyso-NINO-T** would exhibit a strong fluorescence at the same pH value.

Lyso-NINO exhibits high selectivity for NO over other reactive oxygen species and high sensitivity, with a nanomolar-scale limit of detection. **Lyso-NINO** in free form displays a
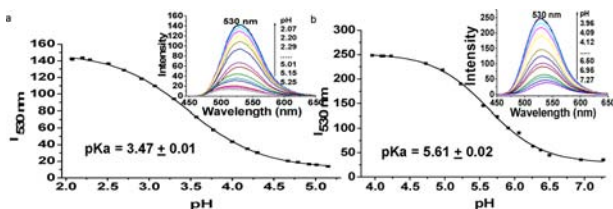
Figure 1. Change of intensity plots of (a) **Lyso-NINO** (11 μM) and (b) **Lyso-NINO-T** (25 μM) at 530 nm vs different pH values. Inset: change in fluorescence spectra of **Lyso-NINO** and **Lyso-NINO-T** in aqueous solution containing 20% acetronitile as cosolvent vs different pH values (λ_{ex} = 440 nm; slit = 3 nm).

broad absorption band centered at 430 nm (ε = 14 500 M^{-1} cm^{-1}) with almost no fluorescence (fluorescence quantum yield Φ = 0.02) in aqueous buffer at pH 5.0. In the presence of 5.0 equiv of NO solution, the emission spectrum of **Lyso-NINO** exhibited a remarkable enhancement (16 times) at 530 nm (Figure S2), and the fluorescence quantum yield increased from 0.02 to 0.3. However, the presence of various reactive oxygen and reactive nitrogen species, such as H_2O_2, NO_2^-, NO_3^-, $^1O_2$, $^\bullet OH$, and $ONOO^-$ (Figure 2a), does not cause an observable
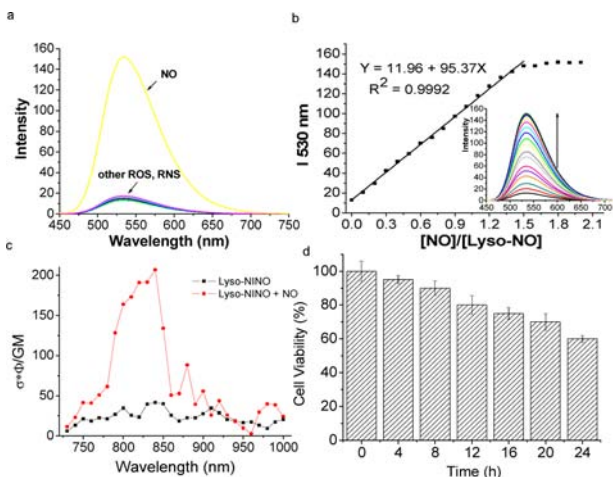

Figure 2. (a) Selectivity of **Lyso-NINO** (11 μM) fluorescence response for NO over other reactive nitrogen and oxygen species (5.0 equiv) in 20:80 CH$_3$CN−H$_2$O solution at pH 5.0 (in 10 mM phosphate buffer). (b) Intensity at 530 nm vs NO concentration under the same conditions. Inset: changes in fluorescence spectra of **Lyso-NINO** (11 μM) upon addition of different amounts NO solution (0−2.0 equiv). (c) Two-photon action spectra of **Lyso-NINO** (in phosphate buffer pH 5.0) before (black) and after (red) addition of NO solution (5.0 equiv). (d) Cell viability of **Lyso-NINO** (5.0 μM) at different times.

spectral change. The titrations of **Lyso-NINO** with NO are examined in a mixture of acetonitrile and sodium phosphate buffer. Upon addition of NO solution, a new emission peak at 530 nm increases gradually (Figure 2b); meanwhile, the absorption spectra do not change during the titration of NO (Figure S3), which indicates that nitric oxide reacts with *o*-phenylenediamine moiety, a result of inhibition of the PET process from the *o*-phenylenediamine moiety to the naphthalimide fluorophore. The *in situ*-formed NO product of **Lyso-NINO** without any byproduct, as expected, is confirmed to be **Lyso-NINO-T**, according to retention times and typical HPLC absorption spectra (Figure S4). With increasing of NO, an excellent linear correlation (R^2 = 0.9992) between fluorescence

intensity (530 nm) and NO concentration is found in the range of NO concentration from 1 to 1.3 μM (Figure 2b). The limit of detection (S/N = 3) of **Lyso-NINO** is determined to be 5 nM (Supporting Information), indicating that **Lyso-NINO** would be a potential tool to monitor endogenous NO in live cells.

Lyso-NINO displays significant applicability under two-photon excitation. The maximum two-photon action cross-section ($\Phi\delta$) value is 34 GM at 840 nm.[13] Upon addition of NO solution (5.0 equiv), the maximum two-photon action cross section increases to 210 GM at 840 nm (Figure 2c). Such a drastic enhancement in two-photon excitation fluorescence indicates that **Lyso-NINO** is a potential two-photon probe for monitoring and imaging NO in living specimens.

The cytotoxicity of **Lyso-NINO** is low in the first 12 h of incubation. MTT assay (SI) has revealed that 80% of MCF-7 cells survive after incubation with **Lyso-NINO** (5.0 μM) for 12 h, and 24 h later the survival rate is over 60% (Figure 2d). As is well known, most lysosomal probes bearing basic groups exhibit an alkalinizing effect on the lysosomes, such that longer incubation with these probes can induce an increase in lysosomal pH and result in cell death. Although there is significant cell death within 24 h incubation time, in the first 12 h the survival rate of these cells incubated with **Lyso-NINO** is high: up to 80%. Compared with some commercial lysosomal probes, the lower cytotoxic effect of **Lyso-NINO** on MCF-7 cells is a favorable characteristic of a practical NO probe for application in living cells.

Lyso-NINO exhibits a significant ability to target lysosomes. Co-localization experiments are performed by co-staining MCF-7 cells with Neutral Red (NR), a commercial LysoTracker, and tetramethylrhodamine methyl (TMRM), a mitochondria tracker. MCF-7 cells stained with **Lyso-NINO** for 10 min at 37 °C do not show green fluorescence in Channel 1 (Figure S5); after that, in the presence of 5.0 equiv of NO solution, these cells display measurable levels of green TP fluorescence in discrete subcellular locations (Figure 3a). The TPM image merges well with the image of staining with NR (Figure 3c) rather than TMRM (Figure S6), indicating that **Lyso-NINO** can specifically localize in lysosomes rather than mitochondria of living cells. The intensity profiles of the linear regions of interest across MCF-7 cells stained with **Lyso-NINO** and NR also vary in close synchrony (Figure 3d). The high Pearson's coefficient and overlap coefficient are 0.805 and 1.374, respectively, evaluated using the conventional dye-overlay method (Figure S7).

Lyso-NINO and NR display a good coexistence in lysosomes of MCF-7 cells, yet the differences in fluorescence intensity between the two channels, which may provide more useful information about the internal environment of organelles in living cells, should be noted. Thus, an intensity correlation analysis (ICA)[14] is employed to assess the intensity distribution of the two co-existing dyes. We plot the intensity of stain **Lyso-NINO** against that of NR for each pixel. The dependent staining in Figure 3c results in a highly correlated plot (Figure 3e), and the ICA plots for the two stains generate an unsymmetrical hourglass-shaped scatterplot that is markedly skewed toward positive values (Figure 3f,g). Moreover, the intensity correlation quotient (ICQ) for the two dyes is 0.357, very close to 0.5, suggesting that the staining intensities are dependent on each other. From the product of the differences from the mean (PDM) image with positive values in the pixels (Figure 3h), we can easily identify those lysosomes with high
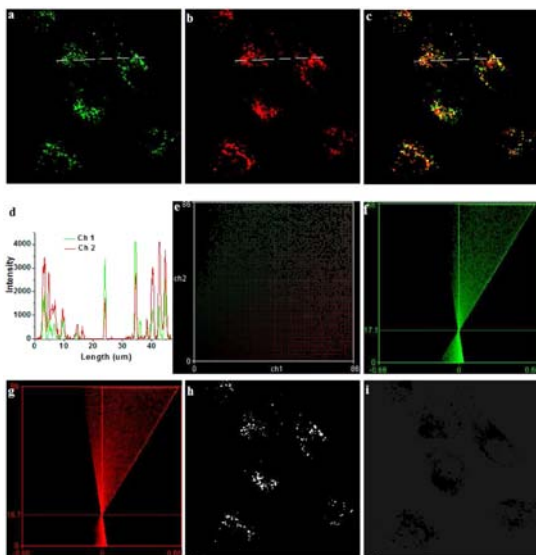
Figure 3. Lyso-NINO co-localizes to lysosomes in MCF-7 cells. MCF-7 was stained with (a) 5.0 μM **Lyso-NINO** with NO solution (20 μM) for 5 min at 37 °C (Channel 1: λ_{ex} = 840 nm, λ_{em} = 520−560 nm) and (b) 5.0 μM NR (Channel 2: λ_{ex} = 559 nm, λ_{em} = 565−610 nm). (c) Overlay of (a) and (b). (d) Intensity profile of regions of interest (ROI) across MCF-7 cells. (e) Intensity correlation plot of stain **Lyso-NINO** and NR. ICA plots of (f) stain **Lyso-NINO**. and (g) stain NR. PDM images with (h) positive PDM values in the pixels and (i) negative PDM values in the pixels.

intensity distribution of the two dyes (white pixels in Figure 3h). There are also some lysosomes for which the intensity distribution does not vary synchronously (Figure 3i). The reason might be that nitrite ions, byproducts of NO, react with NR in acidic conditions and deplete the fluorescence intensity of NR in some lysosomes.[15] On the other hand, the PDM image with negative values in the pixels (Figure 3i) further demonstrates that **Lyso-NINO** is a lysosome-specific probe for NO and the byproduct of NO in lysosomes does not interfere with its photoproperties.

Owing to its high selectivity and sensitivity, **Lyso-NINO** is used as a fluorescent indicator to assess exogenous and endogenous NO in living cells. In view of NO releaser widely used in the treatment of cancer,[16] herein, we employ **Lyso-NINO** to monitor the releasing process of NO from NOC13 in lysosomes of MCF-7 cells. NOC13 (5.0 equiv) is added into MCF-7 cells incubated with **Lyso-NINO**, and the fluorescence intensity gradually increases over time and reaches the maximum after 15 min (Figure 4a). The gradually increasing intensity in lysosomes demonstrates that NO releasing from NOC13 is able to diffuse into lysosomes and then be captured by lyosomes-targeting **Lyso-NINO**. The efficacy of **Lyso-NINO** for endogenous NO is also investigated. Macrophages RAW264.7 incubated with **Lyso-NINO** for 12 h at 37 °C display a weak fluorescence (Figure 4b), which indicates that **Lyso-NINO** could capture endogenous NO in lysosomes of macrophages. Stimulation with L-arginine (L-Arg), interferon-r (IFN-r), and Lipopolysaccharide (LPS) in the presence of **Lyso-NINO** has led to a pronounced increase in fluorescence intensity (Figure 4c), indicating that, despite the shorter lifetime of NO, endogenous NO may easily diffuse into lysosomes in living cells.

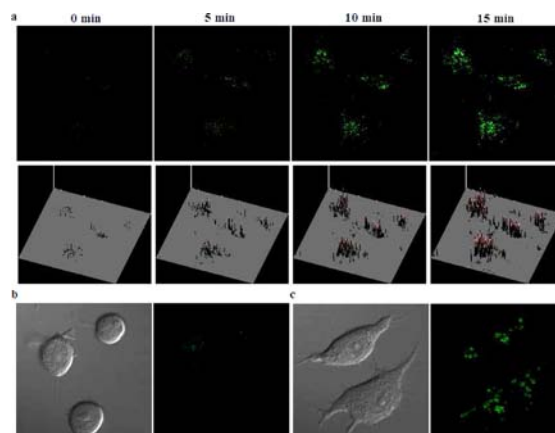**Lyso-NINO** also shows good applicability in flow cytometry (FCM). FCM can be used to quantitatively evaluate

Figure 4. (a) Time-dependent exogenous NO released from NOC13 (20 μM) in MCF-7 cells stained with **Lyso-NINO** (5.0 μM). (b) DIC image and two-photon fluorescent image of RAW 264.7 macrophages stained with **Lyso-NINO** (5.0 μM) for 4 h at 37 °C. (c) DIC image and two-photon fluorescent image of RAW 264.7 macrophages co-stained with **Lyso-NINO** (5.0 μM) and stimulants containing final concentrations of 5000 μg/mL L-Arg, 150 units/mL INF-r, and 20 μg/mL LPS for 12 h, λ_{ex} = 840 nm, λ_{em} = 520−560 nm.

endogenous NO in individual cells on a large scale, compared with microscopy imaging which provides spatial information. Histograms demonstrate intracellular accumulation of NO and increase of intracellular fluorescence indicated by the shift of the fluorescence signal measured in the FITC channel (Figure 5). Compared with control cells without staining (Figure
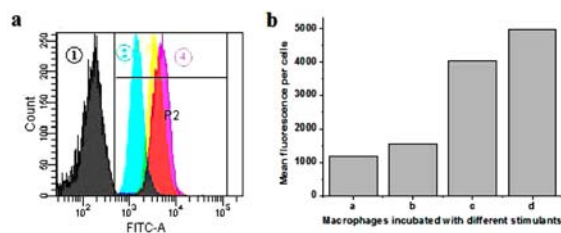

Figure 5. (a) Flow cytometric analysis of Macrophages loaded with **Lyso-NINO** and different stimulants. (1) Control group: Macrophages loaded without **Lyso-NINO**. (2) Macrophages loaded with **Lyso-NINO** (5.0 μM) for 12 h at 37 °C. (3) Macrophages loaded with **Lyso-NINO** (5.0 μM), L-Arg(5000 μg/mL), and LPS (20 μg/mL) for 12 h 37 °C. (4) Macrophages co-incubated with **Lyso-NINO** (5.0 μm), L-Arg(5000 μg/mL), INF-r (150 units/mL), and LPS (20 μg/mL) for 12 h 37 °C. (b) Mean fluorescence per macrophage cell of (1), (2), (3), and (4) incubation conditions.

5a(1)), macrophages incubated only with **Lyso-NINO** for 12 h show a significant fluorescence from the histogram (Figure 5a(2)). Incubation of cells with **Lyso-NINO**, L-Arg, and LPS for 12 h shifts the histogram of green fluorescence in the direction of higher intensity (Figure 5a(3)). However, in the absence and presence of IFN-r, the histograms of fluorescence intensity do not shift much higher (Figure 5a(4)). Quantification by mean fluorescence of these macrophages (Figure 5b) suggests that LPS would be a critical factor which effectively induces an increase of endogenous NO in live macrophages.

In this paper, there are two significant aspects: (1) A lysosomal-specific and two-photon fluorescent probe **Lyso-NINO** has been first developed, and it demonstrates high selectivity and sensitivity toward NO over other reactive oxygen

and nitrogen species, lower cytotoxicity than commercial lysosomal probes, and perfect lysosomal localization. (2) For the first time, endogenous NO has been captured in lysosomes of macrophage cells, despite the unknown NO source. We anticipate that these results would inspire and encourage lots of researchers to focus on the regulation effects of NO on lysosomes in the biology and pharmacology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis, characterization, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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