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Hu, Z., Yang, G., Hu, J., Wang, H., Eriksson, P., Zhang, R., Zhang, Z., Uvdal, K., (2018), Real-time visualizing the regulation of reactive oxygen species on Zn²⁺ release in cellular lysosome by a specific fluorescent probe, *Sensors and actuators. B, Chemical*, 264, 419-425.
<https://doi.org/10.1016/j.snb.2018.02.055>

Original publication available at:

<https://doi.org/10.1016/j.snb.2018.02.055>

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Real-time visualizing the regulation of reactive oxygen species on Zn^{2+} release in cellular lysosome by a specific fluorescent probe

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Graphic Abstract

Abstract

Reactive oxygen species (ROS) regulating the release of free zinc ions (Zn^{2+}) in cellular lysosome is closely related to various pathways of cellular signal transduction, such as inflammation and oxidative stress. Directly visualizing Zn^{2+} release in lysosome is essential for in-depth understanding these physiological processes, and is still an atelic challenge. In this work, we successfully fabricate a lysosome-specific Zn^{2+} fluorescent probe and achieve the visualization of ROS-induced Zn^{2+} release in lysosome of inflammatory cells. The as-prepared probe combines a green fluorophore, an ionophore with five-dentate sites, and a morpholine as the lysosome-specific localization moiety. The fluorescence of the fluorophore in free probe is suppressed by a photoinduced electron transfer (PET) process from nitrogen atoms in the ionophore. Upon the addition of Zn^{2+} , the fluorescence can be promoted immediately, achieving the real-time detection. Meanwhile, the probe is sensitive and selective to Zn^{2+} , which provides the capability to detect low-concentration of free Zn^{2+} in lysosomes. Accordingly, the Zn^{2+} release was clearly observed in lysosome with the increase of ROS levels when the inflammation occurred in living cells.

Introduction

Zinc ion (Zn^{2+}) is an essential element that plays a key role in various fundamental biological processes, such as inflammation, neural signal transmission, cell apoptosis, gene expression, immune function and mammalian reproduction.[1] In the biological environments, zinc is typically found to assemble chelated complex to keep spatial-structure and essential functions of Zn^{2+} -binding biomolecules, including zinc metallothioneins and zinc-finger nucleotide. On the other hand, on the cellular level, there also exists very little amount of free Zn^{2+} to keep the balance between coordinated and free Zn^{2+} in living cells and *in vivo*. The reversible transformation of coordinated and free Zn^{2+} in living cells can regulate zinc-containing proteins/enzymes activities, and thus trigger several important pathways of cellular signal transductions (CST).[2] The precise inter-organelle zinc dynamics within cells warrants further investigation. In other words, real-time visualizing Zn^{2+} dynamics in living cells will be very beneficial for the investigation of the possible involvement of Zn^{2+} in CST procedures.

Actually, CST pathway depends on the cooperation of various subcellular compartments, such as lysosome and mitochondria. As one of the most important metabolic organelles, lysosome contains various acidic hydrolases serving as the main sites for macromolecule degradation. The activities of these hydrolases in lysosome are closely related to the equilibrium of coordinated and free Zn^{2+} , which is involved into many important CST processes. For instance, cellular Zn^{2+} functions as a link between oxidative stress and lysosomal membrane permeabilization (LMP).[3] In inflammation cells,

1 the respiration is accelerated, resulting in excessive ROS generation. A rapid influx of the long-lived
2 ROS such as hydrogen peroxide (H_2O_2) can cause oxidation of the cysteine residues in zinc-binding
3 proteins/enzymes to disulfides and release free Zn^{2+} ions.[4] The released Zn^{2+} then rapidly leak and
4 sequentially accumulate into the lysosomes, and continuously induce downstream bio-events, such
5 as the lysosomal LMP, a potentially lethal event.[5] Therefore, for in-depth understanding CST in the
6 cellular lysosome, how to observe these processes is significant and challenging.

7 To obtain information with high precision from the complicated biological systems, molecular
8 fluorescence imaging is a promising method due to their high sensitivity and high spatial resolution,
9 as well as real-time monitoring capability.[6] So far, many efforts have been made on the
10 developments of Zn^{2+} selective probes for the cellular and subcellular detection of Zn^{2+} . [7-12]
11 However, to enable on-site observation of the process that ROS regulate Zn^{2+} release in lysosome,
12 we do need a specific Zn^{2+} fluorescent probe. In particular, the probe should also meet the following
13 requirements: 1) capability to deliver “turn-on” or ratio fluorescence changes towards Zn^{2+} at weak
14 acidic pH (pH 4.5-5.5 in lysosome); 2) intrinsic sensitivity towards Zn^{2+} due to the fact that most of
15 biological Zn^{2+} existing in coordination complexes, especially substantial amounts are found in the
16 thiol-based proteins; (3) capability to deliver high specificity to lysosome compartment. With these
17 in mind, we conceived a novel way to construct a lysosome-targetable fluorescent probe to reach the
18 proposed Zn^{2+} sensing purpose. The as-prepared probe, **Lys-NBD-TPEA**, is determined to provide a
19 satisfied “turn-on” fluorescence response towards lysosomal Zn^{2+} . Furthermore, **Lys-NBD-TPEA** is
20 then successfully applied to visualize the process of Zn^{2+} release from zinc-binding biomolecules
21 induced by excessive ROS in the inflammatory macrophages.

22 **Experimental section**

23 *2.1. General procedures*

24 Most of the chemicals were AR grade and bought from Sigma-Aldrich. The deionized water was used
25 in all experiments. Proton nuclear magnetic resonance (^1H NMR) and carbon nuclear magnetic
26 resonance (^{13}C NMR) were performed on a Varian 300 MHz spectrometer. Electrospray ionization
27 mass spectra (ESI-MS) were recorded on a Wasters SQ Detector. Preparative liquid chromatography
28 was run on a Gilson Unipoint system with a Gemini C_{18} column under neutral condition using
29 gradient CH_3CN /water buffered with NH_4OAc as eluent (Water phase: 95:5 water/acetonitrile
30 buffered with 10 mM NH_4OAc ; Organic phase: 90:10 acetonitrile/water buffered with 10 mM
31 NH_4OAc). UV-Vis spectra and fluorescence spectra were obtained with Scinco S-4100 UV-Vis
32 Spectrophotometer and Hitachi F-4500 fluorescence spectrometer, respectively, and the pH values
33 were determined by using a PHS-3C meter. All fluorescence measurements were performed at room

temperature. Fluorescent images were acquired on laser confocal microscope (Leica SP8). The excitation laser of **Lys-NBD-TPEA** is 470 nm, and fluorescent images were recorded at the emission range of 500-590 nm. For lyso-tracker, the excitation wavelength is 570 nm, and fluorescent images were recorded at the emission range of 590-670 nm. The resolution of microscope images is 1024 × 1024 pixels.

A stock solution of **Lys-NBD-TPEA** (1 mM) was prepared in DMSO. All the test solutions of **Lys-NBD-TPEA** (10.0 μM) in 50 mM MES (pH 5.0) or 50 mM HEPES (pH 7.4) containing 10% DMSO were prepared. The solutions of various testing species were prepared by diluting the stock solutions.

2.2. Synthesis of **Lys-NBD-TPEA**

Synthesis of 2

The mixture of compound **3** (500 mg, 3.64 mmol), compound **4** (880 mg, 3.64 mmol) and molecular sieves 3 Å in 20 mL of dry ethanol was refluxed overnight under nitrogen atmosphere. Then the reaction mixture was cooled to room temperature and filtered to remove molecular sieves. Sodium borohydride (207 mg, 5.45 mmol) was added to the filtrate in batches. The resulted solution was stirring for another 4 hours at room temperature. The mixture was evaporated in vacuum to dryness. The residue was purified by silica gel flash chromatography with dichloromethane/methanol/ammonium hydroxide (50:10:1) to give light brown oil **2** (1.10 g, 82%). ¹H NMR (300 MHz, CDCl₃): 8.42 (ddd, J = 4.9, 1.7, 0.9 Hz, 2H), 7.63 (t, J = 7.7 Hz, 1H), 7.55 (td, J = 7.7, 1.8 Hz, 2H), 7.30 (d, J = 7.8 Hz, 2H), 7.18 (dd, J = 7.7, 3.7 Hz, 2H), 7.09 (ddd, J = 7.5, 4.9, 1.1 Hz, 2H), 4.69 (s, 2H), 4.00 (s, 2H), 3.84 (s, 4H), 2.94 (s, 4H). ¹³C NMR (75 MHz, CDCl₃): 159.55, 158.76, 154.62, 148.93, 137.38, 136.68, 125.71, 123.33, 122.21, 121.09, 119.57, 64.31, 62.40, 60.22, 52.40, 52.14, 45.55. MS (ESI-MS): Calcd for C₂₁H₂₅N₅O [M+H⁺] 364.21; Found, 364.37.

Synthesis of 1-OH

NBD-Cl (500 mg, 2.50 mmol), compound **2** (824 mg, 2.27 mmol) and trimethylamine (348 μL, 2.50 mmol) were dissolved in 20 mL methanol. The resulted solution was stirring overnight at room temperature. The mixture was concentrated in vacuum. The residue was purified by silica gel flash chromatography dichloromethane/methanol/ammonium hydroxide (100:10:1) to give red solid **1-OH** (894 mg, 75%). ¹H NMR (300 MHz, CDCl₃): 8.46 (ddd, J = 4.9, 1.8, 0.9 Hz, 2H), 8.25 (d, J = 9.0 Hz, 1H), 7.66-7.54 (m, 3H), 7.37 (dd, J = 4.8, 3.8 Hz, 2H), 7.20-7.04 (m, 4H), 6.06 (d, J = 9.1 Hz, 1H), 5.27 (s, 2H), 4.69 (s, 2H), 4.11 (s, 2H), 3.91 (s, 5H), 3.04 (t, J = 6.8 Hz, 2H). ¹³C NMR (75 MHz, CDCl₃): 159.61, 158.43, 154.21, 149.14, 145.09, 144.67, 144.29, 137.71, 136.52, 135.08, 123.41, 122.67, 122.32, 119.83, 119.72, 102.03, 63.94, 60.88, 58.75, 52.21, 50.22. MS (ESI-MS): Calcd for C₂₇H₂₆N₈O₄ [M+H⁺] 527.21; Found 527.32.

1 *Synthesis of Lys-NBD-TPEA*

2 **1-Cl** was synthesized as following identical procedure as described in the synthesis of **NBD-**
3 **TPEA**.^[13] After the complete conversion from **1-OH** (100 mg, 0.19 mmol) to **1-Cl** (determined by
4 ESI-MS, [See in ESI](#)), to the reaction mixture morpholine (33 mg, 0.38 mmol) was added. The resulted
5 solution was further refluxed under nitrogen atmosphere. HPLC-MS was used to monitor the reaction.
6 After the completion of the reaction, the mixture was concentrated in vacuum, and then 10 mL
7 dichloromethane were added. The organic layer was washed with brine, dried over anhydrous sodium
8 sulfide, and evaporated to dryness. The residue was purified by preparative HPLC with a linear
9 gradient (20 min: using initial 20% to final 100% of organic phase) to give **Lys-NBD-TPEA** (83 mg,
10 74 %). ¹H NMR (300 MHz, CDCl₃): δ 8.44 (m, 2H), 8.26 (d, J = 9.0 Hz, 1H), 7.65 (t, 7.53 (m, 3H),
11 7.37 (t, J = 7.7 Hz, 3H), 7.11 (ddd, J = 7.4, 4.9, 1.0 Hz, 2H), 7.00 (d, J = 7.6 Hz, 1H), 6.09 (d, J = 9.0
12 Hz, 1H), 5.22 (br, 2H), 4.19 (br, 2H), 3.92 (s, 4H), 3.75-3.65 (m, 4H), 3.61 (s, 2H), 3.06 (t, J = 6.7
13 Hz, 2H), 2.47 (t, J = 4.5 Hz, 4H). ¹³C NMR (75 MHz, CDCl₃): δ 158.45, 154.56, 149.17, 145.19,
14 144.68, 144.31, 137.41, 136.46, 135.03, 123.31, 122.28, 119.30, 102.16, 66.79, 64.53, 60.75, 59.00,
15 53.61, 52.43, 50.28. MS (ESI-MS): Calcd for C₃₁H₃₃N₉O₄ [M+H⁺] 596.27; Found 596.75.

16 **Results and discussion**

17 *3.1. Design and synthesis of probe Lys-NBD-TPEA*

18 Novel modification of well-known probe substrates to achieve specific probes will greatly simplify
19 the development and facilitate the usage of the probes for different application purposes.^[14] In our
20 previous works, we demonstrated that the extra functional moiety bound to the ionophore has virtually
21 no negative impact on the sensing behaviors of the chemosensors.^[15, 16] For “turn-on” Zn²⁺ sensing,
22 **NBD-TPEA** has been independently developed by two research groups.^[13, 17] In **NBD-TPEA**, 4-
23 amino-7-nitro-2,1,3-benzoxadiazole (ANBD) fluorophore and N,N,N'-tri(pyridin-2-ylmethyl)
24 ethane-1,2-diamine (TPEA) were used as fluorescent reporter and ionophore, respectively. Due to its
25 visible fluorescence, good sensitivity and selectivity to Zn²⁺, **NBD-TPEA** has been commercialized
26 in Sigma Aldrich and continuously used in different applications.^[18-21] Therefore, we strategize a
27 scheme to delivery lysosome-targetable Zn²⁺ probe by suspending morpholine (a lysosome-locating
28 group) onto ionophore TPEA in **NBD-TPEA**. The resulted probe **Lys-NBD-TPEA** should follow a
29 similar sensing behaviors towards Zn²⁺ that confirmed for **NBD-TPEA**, but be endowed with
30 lysosome-specific capability.^[17] The strategy will effectively avoid the more complicated case
31 where we need rebuild the fluorophore to link the targeting moiety for targeting purpose.^[22]

Scheme 1. The synthesis of probe **Lys-NBD-TPEA**

To achieve the proposed probes of **Lys-NBD-TPEA** (Scheme 1), the key compound **1-OH** was firstly synthesized by a similar method described for **NBD-TPEA**.^[17] We only used 6-(hydroxymethyl)-2-pyridinecarbaldehyde (**3**) instead of pyridine-2-aldehyde to react with N-bis-pyridin-2-ylmethylethane-1,2-diamine (**4**) to afford the precursor **2**. To introduce a good leaving group for the further substitute reaction, methanesulfonyl chloride (MsCl) was initially proposed to convert alcohol into mesylate. However, it was found that mesylate was 100% converted to chlorinate at this mild, non-acidic reaction condition.^[23] Certainly, the resulted **1-Cl** can be effectively used to process the further proposed reactions without any negative influences. Even in the resulting mixture of previous step reaction, **1-Cl** could completely react with morpholine to reach the proposed probe compound. As a result, such reaction mixture containing **1-Cl** with the addition of morpholine was further refluxed to deliver **Lys-NBD-TPEA** easily. The yields of the reactions are optimistic and acceptable. The following photophysical measurements demonstrated that **Lys-NBD-TPEA** exhibits poorly fluorescence; and has very similar “turn-on” fluorescent sensing behaviors upon Zn^{2+} compared with **NBD-TPEA**.^[17] It hints that it is a feasible way to develop targeting Zn^{2+} probes by taking advantage of the mature Zn^{2+} probes. The further detailed cellular studies are then arranged to test their targeting abilities.

3.2. Sensing behaviors towards Zn^{2+}

2

3 **Fig. 1** The changes in fluorescence intensity at 536 nm excited at 450 nm (I/I_0) vs pH in 50 mM buffer
 4 solution containing 10% DMSO. I_0 and I represent the fluorescence intensities of **Lys-NBD-TPEA**
 5 (10.0 μM) in the absence and presence of 1 equivalent Zn^{2+} , respectively.

6 To detect Zn^{2+} in the lysosome, an acidic organelle, the first essential step is to determine whether
 7 **Lys-NBD-TPEA** is capable to detect lysosomal Zn^{2+} located in $\sim\text{pH}$ 5.0 environment. As
 8 demonstrated in [Fig. S1 in ESI](#), the fluorescence measurements at various pH values from pH 4.0-8.0
 9 indicate that **Lys-NBD-TPEA** emits poor fluorescence through the photon-induced electrons transfer
 10 (PET) process due to the lone pair electrons of nitrogen atoms in the ionophore. In the presence of
 11 Zn^{2+} , due to the binding Zn^{2+} with dentate nitrogen atoms in the ionophore, the PET process was
 12 mostly suppressed; and the fluorescence of fluorophore was subsequently promoted. As shown in [Fig.](#)
 13 [1](#), we can easily observe that in the presence of Zn^{2+} the effective fluorescence enhancements is ca.
 14 4.5 at pH 5.0 and 10.1 at pH 7.4, respectively. These results imply that the as-prepared probe is
 15 potential to visualize Zn^{2+} at physiological pH as expected. Notably, in the presence or absence of
 16 Zn^{2+} , the fluorescence intensity of the probe at acidic pH is slightly higher than that at neutral pH. It
 17 can be ascribed to the fact that the photoinduced electron transfer (PET) from the nitrogen atom in
 18 morpholine was suppressed by the protonation of nitrogen.[\[24\]](#)

Fig. 2 a) Fluorescent changes of **Lys-NBD-TPEA** (10.0 μ M) at 536 nm excited at 450 nm (I/I_0) to various metal ions in MES buffer (pH 5.0) and HEPES buffer (pH 7.4). I_0 and I represent the fluorescence intensities of **Lys-NBD-TPEA** (10.0 μ M) in the absence and presence of metal ions, respectively; b) time course fluorescent responses at 536 nm (excited at 450 nm) towards 1 equivalent of Zn²⁺ addition in MES buffer (pH 5.0) and HEPES buffer (pH 7.4).

To further evaluate the sensing performance of **Lys-NBD-TPEA** towards Zn²⁺, we test the “turn-on” sensing selectivity of **Lys-NBD-TPEA** towards Zn²⁺ in both pH 5.0 and 7.4 buffer solutions (Fig. 2a). The fluorescent response and the selectivity at pH 7.4 are identical to the profiles of the reported **NBD-TPEA**.^[13, 17] This result demonstrates that the suspended morpholine on the ionophore does not change the geometry configuration and electron structure of the basic **NBD-TPEA**/Zn²⁺ complex as expected. Importantly, selectivity at pH 5.0 shows very similar trends to that at neutral pH (Fig. 2a). These basic studies obviously indicate that **Lys-NBD-TPEA** could cope with specific sensing Zn²⁺ at either neutral pH or acidic lysosomal pH range. When we performed the above photophysical investigations, we noticed that the dynamics of the sensing are fast. To precisely figure out the dynamics between **Lys-NBD-TPEA** and Zn²⁺, the dynamics of fluorescence response of probe towards Zn²⁺ were studied by monitoring the time-dependent fluorescence intensities of probe in the presence of Zn²⁺ at room temperature. As show in Fig. 2b, it is obvious that after addition of 1 equivalent Zn²⁺, the fluorescence intensity at 536 nm is quickly enhanced and remained unchanged within 2 min, which suggested that the probe has very fast dynamics. Therefore, in the subsequent titration experiments (Fig. S2-3 in ESI and Fig. 3), all spectra measurements were performed after the probe exposed to Zn²⁺ for 2 min.

Fig. 3 a) Fluorescence variations of **Lys-NBD-TPEA** (10.0 μM) (excited at 450 nm) under the continuous addition of Zn^{2+} (0.0-16.0 μM) in MES buffer (pH 5.0); b) the plot of intensity at 536 nm versus Zn^{2+} concentrations of the titration of Zn^{2+} (0.0-8.0 μM) in MES buffer (pH 5.0).

Continuous addition of Zn^{2+} to a buffered solution of **Lys-NBD-TPEA** (10 μM) immediately turned the poor-fluorescent solution to green fluorescence due to enhanced emission at 536 nm. Acquisition of fluorescence spectra reveal significant fluorescence “turn-on” without any spectral shifts at pH 5.0 and pH 7.4, respectively (Fig. 3 and S3 in ESI). No spectral shifts are indicative of a fluorescence transition from an identical excited state of ANBD fluorophore, which is in accordance with the reported **NBD-TPEA** for Zn^{2+} sensing.[13, 17] Titration isotherms constructed by total fluorescence intensity as a function of the amount of added Zn^{2+} conforms the 1:1 complexation either at pH 5.0 or pH 7.4 (Fig. 3b and S3b in ESI), which was further confirmed by the Job’s plot results (Fig. S4). The fluorescence intensities at 536 nm were plotted as a function of the concentrations of Zn^{2+} and typical calibrations were obtained ($R^2 = 0.9988$ for pH 5.0 and $R^2 = 0.9981$ for pH 7.0). The linear fluorescence enhancement of **Lys-NBD-TPEA** upon addition of Zn^{2+} were obtained in the range of 0.0-8.0 μM with the detection limits of 4.77×10^{-7} M and 4.25×10^{-7} M ($3\delta/\text{slope}$) at pH 5.0 and pH 7.4 buffer solutions, respectively. In addition, sensing behaviors of **Lys-NBD-TPEA** towards Zn^{2+} exhibit high anti-interference capacities against the other coexistent cations except for ion Cu^{2+} (Fig. S5). Paramagnetic Cu^{2+} significantly reduces the fluorescence intensity, which is the common quenching effect of Cu^{2+} for almost all the reported Zn^{2+} fluorescent probes.[22] This quenching effect does not give any fake fluorescent signal, which will not interfere with Zn^{2+} detection distinctly when the probe is excess. Therefore, by using **Lys-NBD-TPEA**, the detection of intracellular Zn^{2+} was allowed in lysosomes, where the free Zn^{2+} concentration is up to a micromolar level during the certain stimulations, such as inflammation and oxidative stress. [25]

3.3. Imaging

Fig. 4 Fluorescence imaging of probe-loaded A549 cells (scale bar = 20 μ m): a) A549 cells were only incubated with 10 μ M probe for 30 min; b) A549 cells were incubated with 10 μ M probe for 30 min, then sequentially treated with 20 μ M Zn^{2+} for another 30 min, and 5 μ M lysosome tracker for another 10 min; c) A549 cells were incubated with 10 μ M probe for 30 min, and then with 1 mM H_2O_2 for 60 min, and with 5 μ M lysosome tracker for another 10 min (H_2O_2 -induced Zn^{2+} release in A549 cells). d) Green fluorescence intensities recorded from a), b) and c), respectively.

Prior to the biological experiments, we used MTT viability for evaluating the toxic effects of the probe (Fig. S6 in ESI). It demonstrated that the probe is biocompatible and suitable for the further cell studies. After the A549 cells were incubated in 10 μ M **Lys-NBD-TPEA**, there was no obvious fluorescent signal detected in cells, as shown in Fig. 4a. However, when probe-loaded cells were further treated with free Zn^{2+} , the living cells displayed very bright green fluorescence (Fig. 4b),

1 suggesting that our as-prepared probe can be used to visualize free Zn^{2+} in living cells. In our design
2 principle, morpholine was introduced into **Lys-NBD-TPEA** to endow it with the capability of
3 localization in cellular lysosome. Here, we employed lysosome tracker with strong red emission to
4 investigate the localization ability of **Lys-NBD-TPEA** to the lysosome. Fig. 4b shows that green
5 fluorescence from ANBD fluorophore promoted by Zn^{2+} binding well overlapped with the red
6 emission of lysosome tracker (overlap coefficient as high as 0.85), indicating that **Lys-NBD-TPEA**
7 can accumulated into the cellular lysosome as expected. The above results imply that our as-prepared
8 probe has potential capability to monitor the release of free Zn^{2+} from zinc containing bio-molecules
9 in cellular lysosome. As well-known, ROS is a regulator for Zn^{2+} release, and the free Zn^{2+}
10 concentration enhanced with ROS level increasing.[4] As shown in Fig. 4c, we employed H_2O_2 to
11 treat A549 cells, and the cells exhibited strong green fluorescence in cellular lysosome. However,
12 because H_2O_2 is a strong oxidant, it becomes mandatory to test whether chemical effects of H_2O_2
13 promoted these fluorescent changes. Therefore, we measured the fluorescence spectra of **Lys-NBD-**
14 **TPEA** in the absence and presence of 1 mM H_2O_2 at pH 5.0 and 7.0, respectively (Fig. S7 in ESI).
15 The results showed that H_2O_2 had negligible influence on the fluorescence of the probe, as well as
16 Zn^{2+} -induced fluorescence. These results indicated that the green fluorescence from A549 cells (Fig.
17 4c) is upon the changes of lysosomal Zn^{2+} level. It presented a striking contrast with the case that
18 probe-loaded cells did not give any green fluorescence in the absence of H_2O_2 (Fig. 4a). The result
19 suggests that H_2O_2 as a regulator can enhance the level of free Zn^{2+} significantly. Taking the fact
20 together that the probe can detect the supplemented Zn^{2+} (Fig. 4b), we can conclude that **Lys-NBD-**
21 **TPEA** is not only able to track the exogenous Zn^{2+} in the cellular lysosome, but also able to detect
22 the endogenous Zn^{2+} in the presence of H_2O_2 , which releases Zn^{2+} from the cysteine residues by the
23 oxidation. Clearly, probe **Lys-NBD-TPEA** has capability to visual the Zn^{2+} release in the process of
24 cellular signal transductions, such as the inflammation and apoptosis.

Fig. 5 Fluorescence imaging of probe-loaded RAW 264.7 macrophages in the presence and absence of inflammation induced-drug LPS (Scale bar = 20 μ m): a) RAW 264.7 macrophages were incubated with 10 μ M probe for 30 min; b) RAW 264.7 macrophages were sequentially treated with 10 μ M probe for 30 min, and then with 10 μ g/mL LPS for 60 min, and 5 μ M lysosome tracker for another 10 min; c) RAW 264.7 macrophages were co-incubated with the mixture of 10 μ M probe and 1 mM ROS scavenger NAC for 30 min, and then with 10 μ g/mL LPS for 60 min, and 5 μ M lysosome tracker for another 10 min. d) Green fluorescence intensities recorded from a), b) and c), respectively.

Lysosome plays a key role in the processes of inflammation and apoptosis. During such processes, the ROS level in lysosome was obviously enhanced as proven in numerous previous literatures in such processes.[8] Then, we tried to visualize the process of ROS tuning Zn^{2+} release in the inflammatory cells. Firstly, RAW 264.7 macrophages were loaded with probe **Lys-NBD-TPEA** (Fig. 5a), and then treated with lipopolysaccharide (LPS). As shown in Fig. 5c, the bright green fluorescence signals were clearly detected in RAW 264.7 macrophages, which suggests that a large amount of free Zn^{2+} released from zinc-containing bio-molecules. To confirm that Zn^{2+} release was indeed induced ROS, we performed the negative control experiment. After RAW 264.7 macrophages were sequentially treated with probe **Lys-NBD-TPEA**, ROS scavenger N-acetyl-L-cysteine (NAC) and LPS, the green fluorescence was not observed (Fig. 5b). These results indicate that our probe

1 **Lys-NBD-TPEA** can be used to observe the process of ROS tuning Zn^{2+} release in inflammatory the
2 macrophages, which is very helpful for in-depth understanding the roles of ROS and Zn^{2+} in pathway
3 of signal transduction.

4 **Conclusion**

5 In this work, a lysosome-specific Zn^{2+} probe has been readily constructed and successfully
6 investigated. The sensing behavior of **Lys-NBD-TPEA** towards Zn^{2+} demonstrated that the
7 morpholine suspended to the ionophore did not influence the sensing features from the substrate
8 chemosensor. The subsequent bio-detection of Zn^{2+} in two types of cellular lines further confirmed
9 that the induced morpholine moiety endowed the probe with lysosome localization capability as
10 expected. Notably, the as-prepared probe has capabilities to detect both the exogenous and
11 endogenous Zn^{2+} in the lysosome of A549 cell. Further study in the inflammatory macrophages
12 demonstrated that the probe can be applied to real-time observations and hereby track the process of
13 ROS regulating Zn^{2+} in the subcellular lysosome. In general, the work here offers us a feasible
14 strategy to construct organelle-specific probes by suspending functional moieties to some well-known
15 substrate chemosensors, which will greatly reduce the risk of failure on rebuilding a fluorophore to
16 link the targeting moiety, and thus realize different bio-medical detection purposes.

17

18 **Acknowledgements**

19 We acknowledge the supports from the Swedish Research Council (VR) (621-2013-5357), National
20 Natural Science Foundation of China (NO. 21705001 and 21577098), Swedish Government strategic
21 faculty grant in material science (SFO, MATLIU) in Advanced Functional Materials (AFM) (VR Dnr.
22 5.1-2015-5959), Knut and Alice Wallenberg Foundation (KAW) (Dnr. 2012.0083) and Centre in
23 Nano science and Nanotechnology (CeNano). We thank Dr. Xiongyu Wu (Organic Chemistry, IFM,
24 LiU) for discussions and helps in the compound purifications.

25

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