Organic Nanoprobe Cocktails for Multilocal and Multicolor Fluorescence Imaging of Reactive Oxygen Species

Chao Yin, Houjuan Zhu, Chen Xie, Lei Zhang, Peng Chen, Quli Fan,* Wei Huang, and Kanyi Pu*

Hypochlorite (ClO\textsuperscript{−}) as a highly reactive oxygen species not only acts as a powerful “guarder” in innate host defense but also regulates inflammation-related pathological conditions. Despite the availability of fluorescence probes for detection of ClO\textsuperscript{−} in cells, most of them can only detect ClO\textsuperscript{−} in single cellular organelle, limiting the capability to fully elucidate the synergistic effect of different organelles on the generation of ClO\textsuperscript{−}. This study proposes a nanoprobe cocktail approach for multicolor and multiorganelle imaging of ClO\textsuperscript{−} in cells. Two semiconducting oligomers with different π-conjugation length are synthesized, both of which contain phenothiazine to specifically react with ClO\textsuperscript{−} but show different fluorescent color responses. These sensing components are self-assembled into the nanoprobe with the ability to target cellular lysosome and mitochondria, respectively. The mixture of these nanoprobes forms a nano-cocktail that allows for simultaneous imaging of elevated level of ClO\textsuperscript{−} in lysosome and mitochondria according to fluorescence color variations under selective excitation of each nanoprobe. Thus, this study provides a general concept to design probe cocktails for multilocal and multicolor imaging.

1. Introduction

Reactive oxygen species (ROS) are chemical mediators that play crucial roles in signal transductions of living organisms.\[1\]

Among them, hypochlorite (ClO\textsuperscript{−}) is produced by immune cells through the reaction between hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and chloride ions catalyzed by myeloperoxidase (MPO)\[2\] and can serve as a powerful “guarder” in innate host defense to kill invading microbes.\[3\] In addition, ClO\textsuperscript{−} is often upregulated in many inflammatory pathological conditions such as neurodegeneration\[4\], cancer\[5\], cardiovascular diseases\[6\], atherosclerosis\[7\], and rheumatoid arthritis.\[8\] Thereby, imaging of ClO\textsuperscript{−} using activatable fluorophores is critical to understanding the pathological functions of ClO\textsuperscript{−} and developing innovative therapeutic approach to treat these diseases.\[9\]

Lysosome and mitochondria are two major organelles in almost all eukaryotic cells related to the production of ClO\textsuperscript{−}. Lysosome has degradation machinery with abundant enzymes, which contains MPO to catalyze the generation of ClO\textsuperscript{−}\[10\], whereas, mitochondria are the cellular “plants of power” that host respiration chain and are the major sources of ROS including ClO\textsuperscript{−}\[11\]. Although fluorescent molecular probes were developed to detect ClO\textsuperscript{−} in mitochondria or lysosome, most of them only can detect it in one organelle\[10\],\[12\] or in the two organelles separately.\[13\] However, to understand the synergistic effect of lysosome and mitochondria on the production of ClO\textsuperscript{−}, development of probes that can simultaneously image ClO\textsuperscript{−} in both organelles is essential, which remains elusive.

In this contribution, we report a cocktail probe design approach based on organic semiconducting nanoparticles (OSNs) for in vitro multilocal and multicolor imaging of ClO\textsuperscript{−}. OSNs composed of semiconducting polymers or oligomers have recently evolved into a new generation of photonic materials for molecular imaging. The structural versatility of OSNs has led to different biological applications including tumor imaging\[14\], neuroinflammation imaging\[15\], lymph node mapping\[16\], ultrafast hemodynamic imaging\[17\], and neuron activation\[18\]. Although OSNs have been developed for imaging of ROS, selective fluorescence imaging of ClO\textsuperscript{−} has yet to be demonstrated.\[19\]

To make the probe cocktails, two semiconducting oligomers (4 and 5, Scheme 1) and two amphiphilic block copolymers (9 and 10, Scheme 1) are synthesized and used as the building blocks. Both oligomers contain phenothiazine as the...
ClO$^-$ sensing unit but show different multicolor responses toward ClO$^-$; whereas, the copolymers 9 and 10 have morpholine and triphenylphosphine groups to target lysosome and mitochondria, respectively. Nanoprecipitation between the semiconducting oligomer and the amphiphilic copolymer leads to the organelle-targeted nanoprobes: lysosome-targeted nanoprobe (LNP) composed of 4 and 9, and mitochondria-targeted nanoprobe (MNP) composed of 5 and 10. By selective light excitation, the cocktail of LNP and MNP allows for multilocal and multicolor fluorescence imaging of ClO$^-$ in living cells (Figure 1).

2. Results and Discussion

2.1. Synthesis and Characterization

The designs of fluorescence molecular probes for ClO$^-$ sensing are generally based on oxidation and cleavage.[20] For instance, p-methoxyphenol and spirothioether have been used as the reaction site to trigger the probe activation by ClO$^-$.[21] However, fluorescence turn-on signals rather than fluorescence ratiometric signals are generated. To realize multicolor sensing, the phenothiazine-based semiconducting oligomers (4 and 5) were synthesized as shown in Scheme 1. Phenothiazine was first alkylated with 1-bromohexane in presence of sodium hydride (NaH) to obtain compound 1. Bromination of 1 was carried out carefully using N-bromosuccinimide (NBS) to yield a single bromine substituted compound 2. Then 10-hexyl-3-vinyl-10$^H$-phenothiazine (3) was synthesized in 48.1% yield by heating the mixture of 2 and tributyl(vinyl)tin in...

Figure 1. The illustration of organelle-differentiated multilocal and multicolor fluorescence imaging of endogenous ClO$^-$ in macrophage cells using the organic nanoprobe cocktails composed of lysosome-targeted nanoprobe (LNP) and mitochondria-targeted nanoprobe (MNP). LNP targets lysosome and changes its fluorescence color from green to blue upon activation by ClO$^-$; while MNP targets mitochondria and changes its fluorescence color from red to orange upon activation by ClO$^-$.
toluene using Pd(PPh₃)₂Cl₂/2,6-di-tert-butylphenol as catalyst at 100 °C for 24 h. Heck reaction was then conducted between compound 3 and 1,4-dibromobenzene, yielding the semiconducting oligomer 4 (1,4-bis((E)-2-(10-hexyl-10H-phenothiazin-3-yl)vinyl)benzene). The oligomer 5 (4,7-bis((E)-2-(10-hexyl-10H-phenothiazin-3-yl)vinyl)benzo[c][1,2,5]thiadiazole) was synthesized via a similar procedure using compound 3 and 4,7-dibromobenz[a][1,2,5]thiadiazole. To synthesize the organelle-targeted polymers, poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (PEG-b-PPG-b-PEG) was first reacted with 3-bromoprop-1-yne to introduce the terminal alkynyl, yielding 8. Then, click reaction was used to conjugate 8 with 6 or 7 to yield the lysosome-targeted or mitochondria-targeted polymers (9 or 10). The correct chemical structures of all the intermediates and the final products were confirmed by 1H NMR and mass spectra (Supporting Information).

2.2. Optical Responses toward ROS

The optical responses of the nanoprobes toward ROS were investigated. LNP had the maximum absorption peak at 405 nm, which underwent an immediate spectral evolution with addition of ClO⁻. The absorption at 405 nm gradually decreased, concomitant with the emergence of a new blue-shifted band centered at 352 nm (Figure 3a). Accordingly, the solution color changed from yellow green to colorless (Figure 3g). Similarly, MNP exhibited the maximum absorption peak at 503 nm, which gradually blue-shifted to 463 nm with addition of ClO⁻ (Figure 3d), and the solution color changed from red to orange (Figure 3h). LNP and MNP emitted green and red fluorescence with the quantum yields of 0.52 and 0.14, respectively. With the addition of ClO⁻, the emission of LNP gradually decreased at 535 nm, and shifted to 480 nm with the quantum yield of 0.16 (Figure 3b); whereas, the emission of MNP gradually decreased at 635 nm, and shifted to 610 nm with the quantum yield of 0.23 (Figure 3e). Such spectral changes led to the fluorescence multicolor responses of these nanoprobes toward ClO⁻: green to blue for LNP (Figure 3g) and red to orange for MNP (Figure 3h). Furthermore, the fluorescence spectra evolution allowed the signal quantification using the ratiometric fluorescence signals. A good linear correlation between the logarithmic value of ratiometric fluorescence signals (ln(I₄₈₀/I₅₃₅) for LNP and ln(I₆₁₀/I₆₉₀) for MNP) and the concentration of ClO⁻ was observed with the limit of detection of 0.15 × 10⁻⁶ and 0.19 × 10⁻⁶ m for LNP and MNP, respectively (Figure 3c,f).

To investigate the selectivity of the nanoprobes, the ratios of the fluorescence ratiometric signals in the presence of ROS to that in the absence of ROS were calculated and summarized.
Upon the activation of ClO$^{-}$, $I_{480}/I_{535}$ and $I_{610}/I_{690}$ were respectively increased by 14.26- and 5.43-fold, while no obvious changes were detected for other ROS including H$_2$O$_2$, ONOO$^-$, O$_2^-$, 1O$_2$, and •OH. These data demonstrated the high selectivity of LNP and MNP to ClO$^{-}$.

The sensing mechanism of the nanoprobes toward ClO$^{-}$ was studied by testing the reaction product of 4 or 5 after reaction with ClO$^{-}$ using liquid chromatograph mass spectrometer (LC-MS). The results showed an increased molecular weight of $\approx$32 in both compounds (4 and 5) after reaction with ClO$^{-}$ (Figure S11, Supporting Information), which was equal to the molecular weight of oxygen. Thus, it revealed that ClO$^{-}$ mediated oxidation occurred, turning the divalent sulphur into sulfoxide in 4 or 5 (Figure 4). Such ClO$^{-}$ mediated oxidation processes were not susceptible to pH as shown by the pH-independent response of both LNP and MNP toward ClO$^{-}$ (Figure S12, Supporting Information), showing the ability of the nanoprobes to detect ClO$^{-}$ regardless of pH in different organelles.

To enable the multilocal and multicolor imaging of ClO$^{-}$, the optical responses of the LNP/MNP cocktail toward ClO$^{-}$ were then investigated. The weight ratio of LNP to MNP was optimized to be at 7:1, wherein the absorbance of MNP was low and hidden in the absorption peak of LNP (Figure S13, Supporting Information). Selective excitation experiments were conducted to test the ability of differentiating the signals from different probes. Upon excitation at 405 nm for LNP, the single emission peak at 535 nm was observed for the probe in Figure 3i.
cocktail, which gradually changed to the dual peaks at 472 and 590 nm with nearly identical intensities after activation by ClO\(^-\) (20 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}) (\text{Figure 5b}). The spectral evolution of the cocktail toward ClO\(^-\) allowed us to quantify the signals using the ratiometric fluorescence intensity at 472 nm to that at 535 nm (\(I_{472}/I_{535}\)). A good linear correlation between the logarithmic value of ratiometric fluorescence signals (\(\ln(I_{472}/I_{535})\)) and the concentration of ClO\(^-\) was detected with the limit of detection of 0.17 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1} (\text{Figure 5c}). Upon excitation at 570 nm for MNP, the emission peak at 690 nm was observed and gradually blue-shifted to 610 nm after activation by ClO\(^-\) (20 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}) (\text{Figure 5d}). Similarly, a good linear correlation between the logarithmic value of ratiometric fluorescence signals (\(\ln(I_{610}/I_{690})\)) and the concentration of ClO\(^-\) was observed with the limit of detection of 0.19 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1} (\text{Figure 5e}). Upon activation by ClO\(^-\), \(I_{472}/I_{535}\) (\(\lambda_{\text{ex}} = 405 \text{ nm}\)) and \(I_{610}/I_{690}\) (\(\lambda_{\text{ex}} = 570 \text{ nm}\)) were respectively increased by 22.8- and 16.3-fold, while no obvious changes were detected for other ROS including \(\text{H}_2\text{O}_2\), \(\text{ONOO}^-\), \(\text{O}_2^-\), \(\text{O}_2\) and \(\cdot\text{OH}\). This proved the high selectivity of the cocktail toward ClO\(^-\). The excitation-dependent fluorescence ratiometric responses indicated that it is feasible to use the nanoprobe cocktail to image ClO\(^-\) in different cellular organelles according to their fluorescence multicolor changes.

2.3. Validation of Targeting Capability

To examine the subcellular targeting ability, LNP or MNP was incubated with murine macrophage cells (RAW 264.7) and costaining was conducted with LysoTracker Deep Red or MitoTracker Green. It should be noted that only the signals from red channel can be detected from LysoTracker- or MNP-treated cells, while only green fluorescence can be observed from MitoTracker- or LNP-treated cells (Figure S14, Supporting Information). These results confirmed no signal crosstalk between the nanoprobes (LNP and MNP) and their corresponding trackers (LysoTracker and MitoTracker), indicating the feasibility of using these trackers for colocalization studies. As shown in Figure 6, the green fluorescence of LNP and the red fluorescence of MNP overlapped well with the signals from

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**Figure 4.** The proposed reaction mechanism of oligomers a) 4 and b) 5 with ClO\(^-\). The resulted sulfoxide compounds have different fluorescent colors from their corresponding original forms.

**Figure 5.** Optical responses of the LNP/MNP cocktail toward ClO\(^-\). a) Illustration of the fluorescence evolution of the cocktail nanoprobe upon ClO\(^-\) treatment. b) Fluorescence spectra evolution of the cocktail nanoprobe solution (10 \(\mu\text{g mL}^{-1}\)) upon treatment of ClO\(^-\) from 0 to 20 \(\times 10^{-6} \text{ mol} \cdot \text{L}^{-1}\) at the interval of 2 \(\times 10^{-6} \text{ mol} \cdot \text{L}^{-1}\) (\(\lambda_{\text{ex}} = 405 \text{ nm}\)). The fluorescence intensities were normalized at 535 nm. c) The logarithmic value of ratiometric fluorescent signals (\(\ln(I_{472}/I_{535})\)) as a function of ClO\(^-\) concentration. The red line represents linear fitting from \([\text{ClO}^-] = 0 \text{ to } 18 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}\). d) Normalized fluorescence spectra of the cocktail nanoprobe solution (10 \(\mu\text{g mL}^{-1}\)) upon addition of ClO\(^-\) from 0 to 20 \(\times 10^{-6} \text{ mol} \cdot \text{L}^{-1}\) at the interval of 2 \(\times 10^{-6} \text{ mol} \cdot \text{L}^{-1}\) (\(\lambda_{\text{ex}} = 570 \text{ nm}\)). e) The logarithmic value of ratiometric fluorescent signals (\(\ln(I_{610}/I_{690})\)) as a function of ClO\(^-\) concentration. The red line represents linear fitting from \([\text{ClO}^-] = 0 \text{ to } 18 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}\). f) The ratios of ratiometric fluorescence intensity of the cocktail (\(I_{472}/I_{535}\) and \(I_{610}/I_{690}\)) in the presence of ROS to that in the absence of ROS. The error bars represent SDs of three separate measurements.
The nanoprobe cocktail was tested for multilocal and multicolor imaging of ClO$^-$ in murine macrophage cell line (RAW 264.7). To mimic the inflammatory conditions, the cells were pretreated with bacterial cell wall lipopolysaccharide (LPS) and a dimerized soluble cytokine, interferon-γ (IFN-γ) which stimulated resting macrophages to produce ROS including ClO$^-$. The negative control cells were pretreated with a free-radical scavenger, N-acetyl-L-cysteine (NAC), in addition to LPS/IFN-γ to scavenge macrophage-generated ROS. The multilocal and multicolor fluorescence imaging were conducted by selective excitation of LNP at 405 nm and MNP at 570 nm to detect the signals in lysosome and mitochondria, respectively. After incubating the resting macrophage cells with the nanoprobe cocktail, strong fluorescence signals were detected for both the green (520–550 nm) and red (690–750 nm) channels, while almost no fluorescence was observed for the blue (420–460 nm) and orange (580–610 nm) channels under the same condition (Figure 7a). This proved that the nanoprobes remained unactivated state both in lysosome and mitochondria in the resting cells. In contrast, when the cells were stimulated with LPS/IFN-γ prior to incubation with the nanoprobe cocktail, strong fluorescence signals (Figure 7b) were detected for the blue and orange channels. Note that the relatively strong fluorescence in the green channel was caused by the activation of MNP, as the activated MNP could be excited at 405 nm and emit the fluorescence in the range from 500 to 800 nm. Nevertheless, the images in Figure 7b indicated that the nano-cocktails were activated by ClO$^-$ in both lysosome and mitochondria of stimulated macrophage cells. This was also confirmed by reduced signals in both blue and orange channels for the cells cotreated with LPS/IFN-γ and NAC (a ROS scavenger). Furthermore, overlapping the blue and orange channels allowed for simultaneous visualization of the ClO$^-$ distribution in both lysosome and mitochondria (Figure 7b).

The ratiometric fluorescence signals of $I_{\text{Blue}}/I_{\text{Green}}$ (Figure 7d) and $I_{\text{Orange}}/I_{\text{Red}}$ (Figure 7e) of cells treated with LPS/IFN-γ were calculated to be 0.32 and 1.5, respectively. According to the calibration curves in Figure 5c and Figure 5e, the average ClO$^-$ concentrations in lysosomes and mitochondria in activated macrophage cells were estimated to be $=9.0 \times 10^{-6}$ and $8.0 \times 10^{-6}$ M, respectively. Such estimated concentrations were consistent with the values reported in literatures, showing that ClO$^-$ can be generated at $20 \times 10^{-6} - 400 \times 10^{-6}$ M h$^{-1}$ by activated immune cells under inflammatory conditions.[2] These data confirmed the capability of the nanoprobe cocktail for multicolor fluorescence imaging of ClO$^-$ in mitochondria and lysosome in a simultaneous manner. In addition, another nanoprobe cocktail could be made by pairing 4 with 10 and 5 with 9, which exhibited different color variation toward ClO$^-$ (Figure S18, Supporting Information).
3. Conclusion

In conclusion, we have proposed a probe cocktail approach toward multicolor and multiorganelle imaging of ClO− in living cells. The nanoprobe cocktail was made of two organic nanoparticles (LNP and MNP) self-assembled from different pairs of semiconducting oligomers and targeted amphiphilic polymers. Phenothiazine unit within the semiconducting oligomers endowed the nanoprobe specific and sensitive multicolor fluorescent responses toward ClO−; whereas, morpholine and triphenylphosphine groups of the amphiphilic polymers led to the capability of the nanoprobe cocktails to target lysosome and mitochondria, respectively. In combination with good water solubility, small sizes (less than 25 nm), and low cytotoxicity, the nanoprobe cocktail was able to efficiently internalize the particles (LNP and MNP) self-assembled from different pairs of semiconducting oligomers. Phenothiazine unit within the semiconducting oligomers and targeted amphiphilic polymers led to the capability of the nanoprobe cocktails to target lysosome and mitochondria, respectively. In combination with good water solubility, small sizes (less than 25 nm), and low cytotoxicity, the nanoprobe cocktail was able to efficiently internalize the cells, precisely target lysosome and mitochondria, and colorfully delineate the ClO− levels in these organelles with different fluorescent responses. Our study not only provides a new generation of organic nanoparticles that show fluorescence ratiometric responses toward ClO− but also highlights a feasible methodology to image one biomarker in multiple subcellular sites by simply formulating a cocktail of nanoprobe.

4. Experimental Section

Methods: NMR spectra were recorded on a Bruker Ultra Shield Plus 300 MHz NMR. LC-MS was performed on Thermo LCQ Fleet LC-MS with ESI mode (America). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MASS) was performed on a Bruker autoflex under the reflector mode for data acquisition. TEM images were obtained on a JEM 1230 transmission electron microscope with an accelerating voltage of 200 kV. DLS was performed on the Malvern ZetaSizer Nano S. UV–vis spectra were recorded on a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were carried out on a Fluorolog 3-TCSPC spectrofluorometer (Horiba Jobin Yvon). Confocal laser scanning microscope was conducted on an LSM 710 Meta Confocal Scope.

Chemicals: All chemicals were obtained from Sigma-Aldrich unless otherwise stated. LysoTracker Deep Red and MitoTracker Green were purchased from Molecular Probes Company.

10-Heptyl-10H-phenothiazine (1): A 250 mL round-bottom flask with a magnetic stirring bar was charged with a mixture of sodium hydride (60% in mineral oil, 1.35 g, 33.75 mmol) and 1-bromohexane (4.95 g, 30.18 mmol) under an argon atmosphere. Then 5 g of 10H-phenothiazine (dissolved in 25 mL anhydrous DMF) was slowly added into the round-bottom flask. The mixture was stirred at room temperature overnight, which was poured into water and extracted with diethyl ether. The separated organic layer was dried over Na2SO4 and the solvent removed under reduced pressure, the pure product was obtained as a light yellow oil (4.36 g, 75.04%) by column chromatography (silica gel, petroleum ether: diethyl ether = 6.40 g, 90%). 1H NMR (300 MHz, Acetone-d6, ppm) δ: 7.22–7.12 (m, 2H), 7.04–6.90 (m, 4H), 3.93 (t, 2H), 1.84–1.72 (m, 2H), 1.52–1.39 (m, 2H), 1.35–1.22 (m, 4H), 0.86 (t, 3H). ESI-MS m/z: 284.16 [M+H]+.

3-Bromo-10-heptyl-10H-phenothiazine (2): N-Bromosuccinimide (2.85 g, 16.00 mmol) was slowly added to a solution of 1 (4.53 g, 16.00 mmol) in anhydrous DMF (25 mL). The mixture was stirred at room temperature overnight, which was poured into water and extracted with diethyl ether. The separated organic layer was dried over Na2SO4 and the solvent removed using rotary evaporation. The crude product was purified by column chromatography (silica gel, petroleum ether) to afford 1 as a colorless oil (6.40 g, 90%). 1H NMR (300 MHz, Acetone-d6, ppm) δ: 7.22–7.12 (m, 2H), 7.04–6.90 (m, 4H), 3.93 (t, 2H), 1.84–1.72 (m, 2H), 1.52–1.39 (m, 2H), 1.35–1.22 (m, 4H), 0.86 (t, 3H). ESI-MS m/z: 284.16 [M+H]+.
dichloromethane = 20:1). 1H NMR (300 MHz, Acetone-d6, ppm): δ 7.34–7.10 (m, 4H), 7.00–6.83 (m, 3H), 3.86 (t, 2H), 1.80–1.66 (m, 2H), 1.49–1.33 (m, 2H), 1.30–1.19 (m, 4H), 0.83 (t, 3H). ESI-MS m/z: 364.15 [M+H]+.

PEG-b-PPG-b-PEG-10Phenothiazine (3): A Schlenk tube was charged with 3 (200 mg, 0.65 mmol), 1,4-dibromobenzene (76.66 mg, 0.33 mmol), Pd(OAc)2 (8 mg, 0.032 mmol), triphenylphosphine (2.74 g, 1.21 mmol) and P(o-toly)3 (36 mg, 0.176 mmol) before it was sealed with a rubber septum. The Schlenk tube was degassed with three vacuum–argon cycles to remove air. Then anhydrous DMF (0.8 mL) were added to the Schlenk tube, and the mixture was frozen, evacuated and thawed three times to further remove air. The reaction was then conducted at 100 °C for 2 d. After the organic solvent was distilled out, the crude product was purified by column chromatography (silica gel, petroleum ether: dichloromethane = 1:1) to give yellow-green oil (1.64 g, 48.1%). 1H NMR (300 MHz, CDCl3, ppm): δ 7.22–7.09 (m, 4H), 6.94–6.75 (m, 3H), 6.65–6.52 (m, 1H), 5.61 (d, 1H), 5.13 (d, 1H), 3.83 (t, 2H), 1.86–1.73 (m, 2H), 1.48–1.38 (m, 2H), 1.34–1.25 (m, 4H), 0.88 (t, 3H). ESI-MS m/z: 310.24 [M+H]+.

PEG-b-PPG-b-PEG-Triphenylphosphonium (10): Compound 8 (100 mg, 7.87 µmol), compound 7 (6.93 mg, 15.75 µmol), CuSO4·5H2O (4 mg, 0.016 mmol) and sodium ascorbate (1.37 mg, 0.016 mmol) were placed in a 50 mL round-bottom flask under an argon atmosphere. Then anhydrous DMF (10 mL) was injected into the mixture. The reaction was allowed to proceed at room temperature for 2 d. After the solvent was removed, the crude product was suspended in water and purified by dialysis using 3500 Da molecular weight cut-off dialysis membrane. After freeze-drying, 9 (63 mg, 60%) was obtained as grey floccules. 1H NMR (300 MHz, CDCl3, ppm): δ 7.69 (s, 2H), 3.98–3.57 (m, 709H), 3.56–3.44 (m, 100H), 3.43–3.28 (m, 57H), 2.88–2.65 (br, 8H), 2.41–2.22 (br, 4H), 2.09–1.87 (br, 4H), 1.16–1.05 (m, 166H).

PEG-b-PPG-b-PEG-Triphenylphosphonium (10): Compound 8 (100 mg, 7.87 µmol), compound 7 (6.93 mg, 15.75 µmol), CuSO4·5H2O (4 mg, 0.016 mmol) and sodium ascorbate (1.37 mg, 0.016 mmol) were placed in a 50 mL round-bottom flask under an argon atmosphere. Then anhydrous DMF (10 mL) was injected into the mixture. The reaction was allowed to proceed at room temperature for 2 d. After the solvent was removed, the crude product was suspended in water and purified by dialysis using 3500 Da molecular weight cut-off dialysis membrane. After freeze-drying, 10 (74.4 mg, 69.62%) was obtained as grey floccules. 1H NMR (300 MHz, CDCl3, ppm): δ 8.08 (s, 2H), 7.92–7.57 (m, 25H), 3.90–3.58 (m, 783H), 3.56–3.46 (m, 127H), 3.44–3.32 (m, 73H), 1.17–1.07 (m, 202H).

Preparation of Lysosome- or Mitochondria-Targeted Nanoprobe. To synthesize the lysosome-targeted nanoprobe, compound 9 (20 mg) and 4 (0.25 mg) were dissolved in 1.0 mL of THF, and then swiftly dropped into water (10 mL) under sonication. THF was then removed by distillation, blowing on the solution surface under stirring at room temperature. After filtering through a 0.22 µm filter, a bright yellow-green aqueous solution was obtained. The mitochondria-targeted nanoprobe was prepared as a bright red aqueous solution through the similar procedure using compound 10 (20 mg) and 5 (0.25 mg) as the precursors.

Cell Culture: Murine RAW 264.7 cell lines were grown as monolayers in 75 cm2 flasks containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin-glutamine (GIBCO) at 37 °C in a humidified incubator of 5% CO2. Cells were carefully harvested and split when they reached 80% confluence to maintain exponential growth.

Cytotoxicity Test: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were performed to assess the metabolic activity of macrophage cells. The cells were seeded in 96-well plates (Costar, Chicago, IL) at an intensity of 2 × 104 cells mL−1. After 24 h of incubation, the medium was replaced by the nanoprobe solution at the concentration of 5, 10, 15, 20, 25, and 30 µg mL−1, and the cells were then incubated for 24 h. After the designated time intervals, the wells were washed twice with PBS buffer, and 100 µL MTT solution (0.5 mg mL−1) was added to each well. After incubation for 3 h at 37 °C, the MTT medium solution was carefully removed and the formazan crystals were solubilized with 200 µL of dimethylsulfoxide for 15 min. The absorbance value was recorded at 490 nm using a microplate reader.

The absorbance of the untreated cells was used as a control with its absorbance as the reference value for calculating 100% cellular viability.

For determining the cellular localization of the nanoprobe, live RAW 264.7 macrophage cells cultured in 35 mm glass bottom culture dishes were incubated with 20 µg mL−1 nanoprobe (LNP or MNP) for 24 h. Then LysoTracker (1.0 × 10−6 w) or MitoTracker (1.0 × 10−6 w) was added for another 15 min. Then the cells were washed by PBS prior to imaging. To investigate multicolor cellular imaging of different organelles, the experiment can be divided into three groups. For one group, the macrophage cells were cultured with the LNP/MNP nanoprobe cocktail (30 µg mL−1) for 24 h prior to imaging. In the second group, macrophage cells were pretreated with LPS (100 ng mL−1)/IFN-γ (20 ng mL−1) for 24 h, then treated with LNP/MNP nanoprobe.
cocktail (30 µg mL⁻¹) for another 24 h prior to imaging. For the third group, macrophage cells were stimulated with LPS (100 ng mL⁻¹)/IFN-γ (20 ng mL⁻¹) and NAC (1 × 10⁻³ M) for 24 h, then treated with LNPs/MNP nanoprobe cocktail (30 µg mL⁻¹) for another 24 h prior to imaging. All the imaging signals were collected through four channels: blue (420–460 nm, λ_ex = 405 nm), green (520–550 nm, λ_ex = 405 nm), orange (580–610 nm, λ_em = 570 nm), and red (690–750 nm, λ_em = 570 nm).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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