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

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Abstract. Hypochlorite (ClO^-) as a highly reactive oxygen species (ROS) 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^- in cells, most of them can only detect ClO^- in single cellular organelle, limiting the capability to fully elucidate the synergistic effect of different organelles on the generation of ClO^- . We herein propose a nanoprobe cocktail approach towards multicolor and multi-organelle imaging of ClO^- in cells. Two semiconducting oligomers with different π -conjugation length are synthesized, both of which contain phenothiazine to specifically react with ClO^- but show different fluorescent color responses. These sensing components are self-assembled into the nanoprobes with the ability to target cellular lysosome and mitochondria, respectively. The mixture of these nanoprobes forms a nanococktail that allows for simultaneous imaging of elevated level of ClO^- in lysosome and mitochondria according to fluorescence color variations under selective excitation of each nanoprobe. Thus, our 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^-) is produced by immune cells through the reaction between hydrogen peroxide (H_2O_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] Additionally, ClO^- is often upregulated in many inflammatory pathological conditions such as neuron degeneration,^[4] cancer,^[5] cardiovascular diseases,^[6] atherosclerosis^[7] and rheumatoid arthritis.^[8] Thereby, imaging of ClO^- using activatable fluorophores is critical to understanding the pathological functions of ClO^- 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^- . Lysosome has degradation machinery with abundant enzymes, which contains MPO to catalyze the generation of ClO^- ;^[10] whereas, mitochondria are the cellular “plants of power” that host respiration chain and are the major sources of ROS including ClO^- .^[11] Although fluorescent molecular probes were developed to detect ClO^- in mitochondria or lysosome, most of them only can detect it in one organelle^[10b,12] or in the two organelles separately.^[13] However, to understand the synergistic effect of lysosome and mitochondria on the production of ClO^- , development of probes that can simultaneously image ClO^- 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^- . 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^- 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 towards ClO^- ; whereas, the copolymers 9 and 10 have morpholine and triphenylphosphine groups to target lysosome and mitochondria, respectively. Nano-precipitation 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&5) were synthesized as shown in Scheme 1. Phenothiazine was firstly 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 toluene using $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2/2,6\text{-di-}tert\text{-butylphenol}$ 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-10*H*-phenothiazin-3-yl)vinyl)benzene). The oligomer 5 (4,7-bis((*E*)-2-(10-hexyl-10*H*-phenothiazin-3-yl)vinyl)benzo[*c*][1,2,5]thiadiazole) was synthesized *via* a similar procedure using compound 3 and 4,7-dibromobenzo[*c*][1,2,5]thiadiazole. To synthesize the organelle-targeted polymers, PEG-*b*-PPG-*b*-PEG was firstly 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).

Co-nanoprecipitation method was used to synthesize the organelle-targeted nanoprobes. The lysosome- and mitochondria-targeted amphiphilic triblock copolymers (9 and 10) were respectively coprecipitated with the semiconducting oligomers 4 and 5 to obtain LNP and MNP. Transmission electron microscopy (TEM) indicated the spherical morphology for both LNP and MNP (Figures 2a&2d). Dynamic light scattering (DLS) showed the average hydrodynamic sizes of 22.6 ± 0.52 and 24.5 ± 0.86 nm for LNP and MNP, respectively (Figures 2b&2e). The average hydrodynamic diameters of LNP and MNP remained almost unchanged even after storage in phosphate buffered saline (PBS, pH = 7.4) for 30 d (Figures 2c&2f), indicating the high stability of the nanoprobes probably due to good water-solubility provided by PEG segment from the amphiphilic copolymers as well as strong hydrophobic interactions between the PPG segment and the semiconducting oligomer.

2.2. Optical Responses towards ROS

The optical responses of the nanoprobes towards 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 a shoulder peak at 450 nm with the quantum yield of 0.16 (Figure 3b); whereas, the emission of MNP gradually decreased at 690 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 nanoprobe towards 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 ($\ln(I_{480}/I_{535})$ for LNP and $\ln(I_{610}/I_{690})$ for MNP) and the concentration of ClO^- was observed with the limit of detection of 0.15 and 0.19 μM for LNP and MNP, respectively (Figures 3c&3f).

To investigate the selectivity of the nanoprobe, the ratios of the fluorescence ratiometric signals in the presence of ROS to that in the absence of ROS were calculated and summarized in Figure 3i. 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_2O_2 , ONOO^- , O_2^- , $^1\text{O}_2$, and $\bullet\text{OH}$. These data demonstrated the high selectivity of LNP and MNP to ClO^- .

The sensing mechanism of the nanoprobe towards 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 *ca.* 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 towards ClO^- (Figure S12, Supporting Information), showing the ability of the nanoprobe 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 towards 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 cocktail, which gradually changed to the dual peaks at 472 and 590 nm with nearly identical intensities after activation by ClO^- (20 μM) (Figure 5b). The spectral evolution of the cocktail towards ClO^- allowed us to quantify the signals using the ratiometric fluorescence intensity at 472 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 μM (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 μM) (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 μM (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 H_2O_2 , ONOO^- , O_2^- , $^1\text{O}_2$, and $\bullet\text{OH}$. These results proved the high selectivity of the cocktail towards 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 co-staining 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 both nanoprobe (LNP & MNP) and their corresponding trackers (LysoTracker & 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 LysoTracker and MitoTracker, respectively. The changes in the intensity profiles of linear regions of interest between LNP and LysoTracker (Figure 6b) or MNP and MitoTracker (Figure

6e) were also synchronous and well-overlapped. Furthermore, the intensity of the correlation plot (Figures 6c&6f) reflected high overlap coefficients for LysoTracker & LNP (0.90) and MitoTracker & MNP intensities (0.84). These data demonstrated the excellent intracellular targeting capabilities of LNP and MNP towards lysosome and mitochondria, respectively. In combination with the specific responses towards ClO^- and good cytocompatibility (Figure S15, Supporting Information), the nanoprobe should have the potential for ClO^- imaging at the subcellular level.

2.4. Multilocal and Multicolor Imaging of ClO^-

The nanoprobe cocktail was tested for multilocal and multicolor imaging of endogenous ClO^- in murine macrophage cell line (RAW 264.7). To mimic the inflammatory conditions, the cells were pre-treated 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 pre-treated 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 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 nanoprobe 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 nanococktails 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 co-treated 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. Thus, 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 and $8.0 \mu\text{M}$, respectively. Such estimated concentrations were consistent with the values reported in literatures, showing that HClO can be generated at 20-400 μM per hour by activated neutrophils under inflammatory conditions.^[2] Thus, our nanoprobe cocktail was able to visualize ClO^- at the subcellular level.

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 towards ClO^- (Figure S18, Supporting Information).

3. Conclusion

In conclusion, we have proposed a probe cocktail approach towards multicolor and multi-organelle 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 towards 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 cells, precisely target lysosome and mitochondria, and colorfully delineate the ClO^- levels in these organelles with different fluorescence responses. Our study not only provides a new generation of organic nanoparticles that show fluorescence ratiometric responses towards ClO^- but also highlights a feasible methodology to image one biomarker in multiple subcellular sites by simply formulating a cocktail of nanoprobess.

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. Dynamic light scattering (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 (CLSM) 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-Hexyl-10H-phenothiazine (I): A 250 mL round-bottom flask with 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 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- d_6 , ppm) δ : 7.22-7.12 (m, 4H), 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 $[\text{M}+\text{H}]^+$.

3-Bromo-10-hexyl-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 organic layer was washed with brine, and then dried over Na_2SO_4 . After the solvent was 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: dichloromethane = 20:1). ^1H NMR (300 MHz, Acetone- d_6 , 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 $[\text{M}+\text{H}]^+$.

10-Hexyl-3-vinyl-10H-phenothiazine (3): **2** (4.00 g, 11.02 mmol), tributyl(vinyl)tin (4.21 g, 13.22 mmol), $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ (0.685 g, 0.97 mmol) and 2, 6-di-*tert*-butylphenol (95 mg, 0.46 mmol) were placed in a 50 mL round-bottom flask and the reaction vessel was degassed and purged with N_2 . Then 20 mL of anhydrous toluene was injected into the mixture, which was subjected to three freeze-pump-thaw cycles to remove O_2 . The mixture was vigorously stirred at 100 °C for 24 h. 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, CDCl_3 , 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 $[\text{M}+\text{H}]^+$.

1,4-Bis((E)-2-(10-hexyl-10H-phenothiazin-3-yl)vinyl)benzene (4): A Schlenk tube was charged with **3** (200 mg, 0.65 mmol), 1,4-dibromobenzene (76.66 mg, 0.33 mmol), $\text{Pd}(\text{OAc})_2$ (8 mg, 35.64 μmol), and $\text{P}(o\text{-tolyl})_3$ (36 mg, 117.6 μmol) before it was sealed with a rubber septum. The Schlenk tube was degassed with three vacuum-argon cycles to remove air. Then, DMF (3 mL) and triethylamine (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 $^\circ\text{C}$ for 2 days. After the organic solvent was distilled out, the crude product was purified by column chromatography (silica gel, petroleum ether: dichloromethane = 1:1) to afford yellow-green oil (94.58 mg, 41.4%). ^1H NMR (300 MHz, CDCl_3 , ppm) δ : 7.45 (s, 4H), 7.32-7.27 (m, 4H), 7.13 (d, 4H), 7.00-6.77 (m, 10H), 3.84 (t, 4H), 1.87-1.75 (m, 4H), 1.47-1.39 (m, 4H), 1.35-1.28 (m, 8H), 0.88 (t, 6H). ESI-MS m/z : 692.36 $[\text{M}]^+$.

4,7-Bis((E)-2-(10-hexyl-10H-phenothiazin-3-yl)vinyl)benzo[c][1,2,5]thiadiazole (5): Compound **5** was synthesized by a similar procedure with compound **4** using 4,7-dibromobenzo[c][1,2,5]thiadiazole instead of 1,4-dibromobenzene as the starting material. Yield: 38.2%. ^1H NMR (300 MHz, THF-d_8 , ppm) δ : 8.02 (d, 2H), 7.73 (s, 2H), 7.56 (d, 2H), 7.46-7.40 (m, 4H), 7.15-7.07 (m, 4H), 6.96 (t, 4H), 6.88d (t, 2H), 3.93 (t, 4H), 1.85-1.76 (m, 4H), 1.52-1.42 (m, 4H), 1.34-1.27 (m, 8H), 0.88 (t, 6H). MALDI-TOF, m/z : Calcd, 750.29; Found, 750.53 $[\text{M}]^+$.

4-(3-Azidopropyl)morpholine (6): Sodium azide (0.78 g, 12 mmol) was added to a solution of 4-(3-bromopropyl)morpholine (0.5 g, 2.42 mmol) in anhydrous DMF (20 mL) and the mixture was stirred at room temperature for 24 h. After the solvent was removed by rotary evaporation, the mixture was extracted with water and dichloromethane. The pure product can be obtained without further purification after removing the solvent of organic layer (0.39 g, 94.66%). ¹H NMR (300 MHz, CDCl₃, ppm) δ: 3.70 (t, 4H), 3.34 (t, 2H), 2.51-2.33 (m, 6H), 1.80-1.71 (m, 2H). ESI-MS m/z: 171.03 [M+H]⁺.

(4-Azidobutyl)triphenylphosphonium bromide (7): Sodium azide (153 mg, 2.35 mmol) was added to a solution of (4-bromobutyl)triphenylphosphonium bromide (0.73 g, 1.53 mmol) in anhydrous DMF (15 mL) and the mixture was stirred at room temperature for 24 h. After the solvent was removed by rotary evaporation, dichloromethane was added to the mixture and the precipitate was removed. The product was obtained as white crystals after distilling dichloromethane (0.61 g, 89.97%). ¹H NMR (300 MHz, CDCl₃, ppm) δ: 7.93-7.59 (m, 15H), 3.89 (t, 2H), 3.42 (t, 2H), 2.10-1.89 (m, 2H), 1.80-1.58 (m, 2H). ESI-MS m/z: 360.12 [M-Br]⁺.

PEG-b-PPG-b-PEG-alkyne (8): PEG-*b*-PPG-*b*-PEG (1 g, 0.079 mmol), sodium hydride (60% in mineral oil, 38 mg, 0.95 mmol) and 10 mL anhydrous THF were placed in a 50 mL round-bottom flask and the mixture kept stirring for 1 h. Then 250 μL 3-bromoprop-1-yne (80% in toluene) was added and the mixture was stirred at room temperature for 24 h. After the precipitate was filtered away, the mixture was concentrated and poured into diethyl ether. The precipitate was collected and

redissolved in water. Finally, the product was purified by dialysis against distilled water for 2 days using a 5000 Da molecular weight cut-off dialysis membrane. After freeze-drying, **8** (0.65 g, 65%) was obtained as white floccules. ^1H NMR (300 MHz, CDCl_3 , ppm) δ : 4.20 (d, 4H), 3.91-3.58 (m, 783H), 3.56-3.45 (m, 106H), 3.44-3.24 (m, 61H), 2.44 (t, 2H), 1.21-1.04 (m, 167H).

PEG-b-PPG-b-PEG-morpholine (9): Compound **8** (105 mg, 8.26 μmol), compound **6** (5.6 mg, 32.94 μmol), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (8 mg, 0.032 mmol) and sodium ascorbate (6.54 mg, 0.032 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 days. 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, CDCl_3 , 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), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (4 mg, 0.016 mmol) and sodium ascorbate (3.17 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 days. 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, CDCl_3 , 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 nitrogen 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 mono-layers in 75- cm^2 flasks containing DMEM supplemented with 10% FBS, 1% penicillin streptomycin glutamine (GIBCO) at 37 $^\circ\text{C}$ in a humidified incubator of 5% CO_2 . Cells were carefully harvested and split when they reached 80% confluence to maintain exponential growth.

Cytotoxicity test: 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 2×10^4 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 $\mu\text{g mL}^{-1}$, 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 μ L of dimethylsulfoxide (DMSO) 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.

Cellular imaging: For determining the subcellular location of the nanoprobe, live RAW 264.7 macrophage cells cultured in 35-mm glass bottom culture dishes were incubated with 20 μ g mL⁻¹ nanoprobe (LNP or MNP) for 24 h. Then LysoTracker (1.0 μ M) or MitoTracker (1.0 μ M) 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 μ g 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 μ g 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 mM) for 24 h, then treated with LNP/MNP nanoprobe cocktail (30 μ g 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_{ex} = 570 nm) and red (690-750 nm, λ_{ex} = 570 nm).

Supporting Information

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

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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^- .

Scheme 1. Design and synthesis of the phenothiazine-based semiconducting oligomers (4&5) and the amphiphilic block copolymers (9&10) that target the lysosome and mitochondria, respectively. Reaction conditions: (i) 1-bromohexane, NaH, anhydrous DMF, room temperature. (ii) NBS/anhydrous DMF, room temperature. (iii) tributyl(vinyl)tin, Pd(PPh₃)₂Cl₂/2,6-di-tert-butylphenol, anhydrous toluene, 100 °C. (iv) Pd(OAc)₂/P(*o*-tolyl)₃, anhydrous DMF, triethylamine, 100 °C. (v) Pd(OAc)₂/P(*o*-tolyl)₃, anhydrous DMF, triethylamine, 100 °C. (vi) Sodium azide, anhydrous DMF, room temperature. (vii) Sodium azide, anhydrous DMF, room temperature. (viii) NaH, anhydrous THF, 3-bromoprop-1-yne, room temperature. (ix) CuSO₄•5H₂O, sodium ascorbate, anhydrous DMF, room temperature. (x) CuSO₄•5H₂O, sodium ascorbate, anhydrous DMF, room temperature.

Figure 2. Characterization of the lysosome-targeted nanoprobe (LNP) and mitochondria-targeted nanoprobe (MNP). a) Representative transmission electron microscopy (TEM) of LNP. b) Dynamic light scattering (DLS) of the LNP. c) Average hydrodynamic diameters of the LNP stored in PBS for different time periods. d) Representative TEM of MNP. e) DLS result of the MNP. f) Average hydrodynamic diameters of the MNP stored in PBS for different time periods. The error bars represent standard deviations (SD) of three separate measurements.

Figure 3. Optical responses of the lysosome- and mitochondria-targeted nanoprobe (LNP and MNP) towards ROS. a) UV-vis absorption spectra of LNP ($10 \mu\text{g mL}^{-1}$) upon addition of ClO^- from 0 to $20 \mu\text{M}$ at the interval of $2 \mu\text{M}$. b) Gradual evolution of fluorescence spectra of LNP solution ($10 \mu\text{g mL}^{-1}$) upon treatment of ClO^- from 0 to $20 \mu\text{M}$ at the interval of $2 \mu\text{M}$ ($\lambda_{\text{ex}} = 405 \text{ nm}$). c) The logarithmic value of ratiometric fluorescence signals of LNP ($\ln(I_{480}/I_{535})$) as a function of ClO^- concentration. The red line represents linear fitting from $[\text{ClO}^-] = 0$ to $18 \mu\text{M}$. d) UV-vis absorption spectra of MNP ($10 \mu\text{g mL}^{-1}$) upon addition of ClO^- from 0 to $20 \mu\text{M}$ at the interval of $2 \mu\text{M}$. e) Fluorescence spectra of MNP solution ($10 \mu\text{g mL}^{-1}$) upon addition of ClO^- from 0 to $20 \mu\text{M}$ at the interval of $2 \mu\text{M}$ with the excitation at 450 nm . f) The logarithmic value of ratiometric fluorescence signals of MNP ($\ln(I_{610}/I_{690})$) as a function of ClO^- concentration. The red line represents linear fitting from $[\text{ClO}^-] = 0$ to $20 \mu\text{M}$. g) Photographs of the LNP solution before and after addition of ClO^- under ambient light and a black lamp ($\lambda = 365 \text{ nm}$). h) Photographs of the MNP solution before and after addition of ClO^- under ambient light and a black lamp ($\lambda = 365 \text{ nm}$). i) The ratios of ratiometric fluorescence intensity of LNP (I_{480}/I_{535}) and MNP (I_{610}/I_{690}) in the presence of ROS to that in the absence of ROS. The error bars represent standard deviations (SD) of three separate measurements.

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

Figure 5. Optical responses of the LNP/MNP cocktail towards ClO^- . a) Illustration of the fluorescence evolution of the cocktail nanoprobes upon ClO^- treatment. b) Fluorescence spectra evolution of the cocktail nanoprobes solution ($10 \mu\text{g mL}^{-1}$) upon treatment of ClO^- from 0 to $20 \mu\text{M}$ at the interval of $2 \mu\text{M}$ ($\lambda_{\text{ex}} = 405 \text{ nm}$). The fluorescence intensities were normalized at 535 nm . c) The logarithmic value of ratiometric fluorescence signals ($\ln(I_{472}/I_{535})$) as a function of ClO^- concentration. The red line represents linear fitting from $[\text{ClO}^-] = 0$ to $18 \mu\text{M}$. d) Normalized fluorescence spectra of the cocktail nanoprobes solution ($10 \mu\text{g mL}^{-1}$) upon addition of ClO^- from 0 to $20 \mu\text{M}$ at the interval of $2 \mu\text{M}$ ($\lambda_{\text{ex}} = 570 \text{ nm}$). e) The logarithmic value of ratiometric fluorescence signals ($\ln(I_{610}/I_{690})$) as a function of ClO^- concentration. The red line represents linear fitting from $[\text{ClO}^-] = 0$ to $18 \mu\text{M}$. 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.

Figure 6. Intracellular localization of LNP and MNP in macrophage cells. a) Images of macrophage cells incubated with LNP ($20 \mu\text{g mL}^{-1}$) for 24 h, and subsequently LysoTracker ($1.0 \mu\text{M}$) for another 15 min. Red, LysoTracker fluorescence; green, LNP fluorescence; yellow, merged signal. b) Intensity profile of linear regions of interest across macrophage cells (white arrow in Figure 6a). Red and green lines represent the intensity of the LysoTracker and LNP, respectively. c) Correlation plot of LysoTracker and LNP intensities. d) Images of macrophage cells treated with MNP ($20 \mu\text{g mL}^{-1}$) for 24 h, and subsequently MitoTracker ($1.0 \mu\text{M}$) for another 15 min. Green, MitoTracker fluorescence; Red, MNP fluorescence; yellow, merged signal. e) Intensity profile of linear regions of interest across macrophage cells (white arrow in Figure 6d). Red and green lines represent the intensity of the MNP and MitoTracker, respectively. f) Correlation plot of MitoTracker and MNP intensities.

Figure 7. Multilocal and multicolor imaging of ClO^- in murine macrophage cells (RAW 264.7). a) RAW 264.7 cells were incubated with the nanoprobe cocktail ($30 \mu\text{g mL}^{-1}$) for 24 h without any pretreatment. b) Cells were pretreated with LPS (100 ng mL^{-1})/IFN- γ (20 ng mL^{-1}) for 24 h and then incubated with the nanoprobe cocktail ($30 \mu\text{g mL}^{-1}$) for another 24 h. c) Cells were pretreated with LPS (100 ng mL^{-1})/IFN- γ (20 ng mL^{-1}) and NAC (1 mM) for 24 h, and then incubated with the nanoprobe cocktail ($30 \mu\text{g mL}^{-1}$) for another 24 h. All the imaging signals were collected through four channels: blue ($420\text{--}460 \text{ nm}$, $\lambda_{\text{ex}} = 405 \text{ nm}$), green ($520\text{--}550 \text{ nm}$, $\lambda_{\text{ex}} = 405 \text{ nm}$), orange ($580\text{--}610 \text{ nm}$, $\lambda_{\text{ex}} = 570 \text{ nm}$) and red ($690\text{--}750 \text{ nm}$, $\lambda_{\text{ex}} = 570 \text{ nm}$). d) Quantification of the ratiometric fluorescence signals of the blue channel to that of the green channel ($I_{\text{Blue}}/I_{\text{Green}}$) and their logarithmic values ($\ln(I_{\text{Blue}}/I_{\text{Green}})$) of the macrophage cells upon different treatments: control, +LPS/IFN- γ , and +LPS/IFN- γ +NAC. e) Quantification of the ratiometric fluorescence signals of the orange channel to that of the red channel ($I_{\text{Orange}}/I_{\text{Red}}$) and their logarithmic values ($\ln(I_{\text{Orange}}/I_{\text{Red}})$) of the macrophage cells upon different treatments: control, +LPS/IFN- γ , and +LPS/IFN- γ +NAC. The error bars represent standard deviations (SD) of three separate measurements.

The table of contents entry should be 50–60 words long

Organic nanoprobe cocktails are developed based on two semiconducting oligomers with different fluorescence color responses towards ClO^- . The nanococktails allow for simultaneous and differential multicolor imaging of elevated levels of ClO^- in cellular lysosome and mitochondria.

Keyword: nanoparticles, activatable probe, reactive oxygen species, fluorescence imaging

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

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