Mitochondrion Targeting Fluorescent Probe for Imaging of Intracellular Superoxide Radical

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Supporting Information

1. General Information

UV-Vis absorption spectra were recorded with Varian Cary 50 Bio UV/Vis spectrophotometers (Varian, Inc., Palo Alto, CA) using 1cm quartz disposable cuvettes. UV-Vis absorption strength and fluorescence intensity were measured on Perkin Elmer Wallac 1420 Victor 2 Microplate Reader (PerkinElmer, Inc., Waltham, MA). Fluorescence spectra was obtained by using Spex Fluorolog Tau-3 fluorescence spectrophotometer (Horiba Scientific, Edison, NJ).^{1}H NMR (400 MHz) spectra were recorded on Varian Unity Inova spectrometers (Varian, Inc., Palo Alto, CA). MS spectra were measured by Agilent 1200 series 6210 Time-of-Flight LC/MS mass spectrometer (Agilent technologies, Santa Clara, CA). The confocal laser scanning microscopy (CLSM) was performed on Leica SP5 Inverted Confocal Microscope (Leica Microsystems, Inc., Buffalo Grove, IL) with a 40×H_2O-immersion objective lens. Cells were incubated with Thermo Scientific™ Forma™ Steri-Cult CO_2 Incubator (Thermo Fisher Scientific Inc., Waltham, MA)

Sodium Peroxynitrite was from Cayman chemical Inc. (Ann Arbor, MI). Mitochondrial locating signal (MLS) peptides (NH2-MSVLTPLLLRGLTGSARRLPVPRAK-COOH) were obtained from Genemed synthesis Inc. (San Antonio, TX) with a high purity (>95%). Mitotracker Red CMXRos was from Cell signaling technology (Beverly, MA). H_2O_2 probe peroxy orange 1 (PO1) was from Tocris™ Bioscience (Boston, MA). KO_2 was from Fisher Scientific (Hampton, NH). All other chemicals including Fluorescein isothiocyanate (FITC), 2,4-dinitrobenzenesulfonyl chloride, triethylamine(Et_3N),SWCNT-COOH, glutathione, 2-methoxyestradiol (2-ME), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Phorbol 12-myristate 13-acetate (PMA) etc. were obtained from Sigma Aldrich (St. Louis, MO).

2. Experimental Procedures

Synthesis and identification of Bis (2,4-dinitrobenzenesulfonyl) fluorescein isothiocyanate (1).

Et_3N (0.3 ml, 2.96 mM) was gradually added to a suspension of FITC (0.53 g, 1.36 mM) in CH_2Cl_2 (20 ml) at 0℃ and stirred. Following the 2, 4-dinitrobenzenesulfonyl chloride (0.8 g,

3 mM) was added and the reaction mixture was stirred overnight. The reaction was monitored by TLC under UV light at 254 and 365 nm. After completion of reaction, the mixture was evaporated to remove the solvent, followed by a column chromatography purification using dichloromethane /acetone (40:1). Then the residue was subject to recrystallization to afford 1 as a yellow solid (0.47 g, 40.6% yield).

After the preparation, the product was identified by ^{1}H NMR (400 MHz) spectra and LC-MS spectra.

Preparation and characterization of MLS-1

To a MLS peptide stock solution (0.2mM) prepared in carbonate-bicarbonate buffer (0.1 M, pH 9.0±0.1), add DMSO stock solution of **1** dropwise with a pipette (0.1-2.5 μl) while stirring to the reaction vial to make the final concentration 0.1 mM. The reaction solution should be protected from light and incubated for 2 h at room temperature with gentle stirring.

After the preparation, remove the salt in MLS-**1** solution with pipette tips. Apply HPLC analysis with acetonitrile (0.1% TFA) and deionized water (0.1% TFA) in a ratio of 80:20. 20 μl of the sample solution (200 μM MLS) was injected into the system and then eluted over 30 minutes at a flow rate of 100 μl/min. The column temperature was kept constant at 25 ℃.

Reaction rate of 1 toward $O_2^{-}\cdot$

Various concentrations of KO_2 (0.65, 6.5, 13, 25, 50, 100 μM) were respectively added in the phosphate buffer (50 mM, pH 7.4) containing **1**/MLS-**1**(10μM). Observe the fluorescence intensity of **1**/MLS-**1** solution 3, 5, 10, 20, 40 or 60 min after the reaction at EX/EM (494/520 nm) in a black 96-Well Immuno Plate.

Selectivity of 1/MLS-1 toward ROS/RNS and reagents in solution

H_2O_2, $ONOO^{-}$, GSH, ClO^{-}, Fe^{2+} and Fe^{3+} solutions were prepared by directly diluting commercially available H_2O_2 (50 μM), NaONOO (50 μM), GSH (100 μM), NaClO (100 μM), $FeSO_4$ (100 μM), and $FeCl_3$ (100 μM). $O_2^{-}\cdot$ was generated from KO_2 (25 μM). HO· (50 μM) and $^{1}O_2$ (50 μM) were respectively produced from Fenton reaction (H_2O_2-$FeSO_4$) system and H_2O_2-molybdate ions (Na_2MoO_4) system. The fluorescence intensity of **1**/MLS-**1** solution (10 μM) in 50 mM phosphate buffer (pH 7.4) were detected at EX/EM (494/520 nm) 40 min after addition of above ROS/RNS and reagents in a black 96-Well Immuno Plate.

Sensitivity of 1/MLS-1 toward $O_2^{-}\cdot$

Different concentrations of KO_2 (0.65, 1.3, 2.6, 6.5, 13, 25 μM) were respectively added in the phosphate buffer (50 mM, pH 7.4) containing **1**/MLS-**1**(10 μM). Observe the fluorescence

intensity of **1**/MLS-**1** solution 10min after the reaction at EX/EM (494/520 nm) in a black 96-Well Immuno Plate.

Response of 1/MLS-1 toward $O_2^{-}\cdot$

Excess KO_2 (25 μM) were respectively added in phosphate buffer (50 mM, pH 7.4) containing various concentrations of **1**/MLS-**1** (0.1, 0.25, 0.5, 1, 2.5 μM) in a black 96-Well Immuno Plate to ensure complete reaction. The fluorescence intensity of **1**/MLS-**1** solution (10 μM) were detected at EX/EM (494/520 nm) 10 min after addition of KO_2.

Cell culture

The Raw 264.7 macrophages cell line (InvivoGen Inc.) or Hela ATCC® CCL-2™ cell line (ATCC) were grown in DMEM medium supplemented with 10% FBS (Fetal bovine serum) and 1% penicillin at 37 ℃ in humidified environment containing 5% CO_2. Cells were plated on 24-well or 96-well microtiter plate and allowed adhere for 24 hours.

Confocal Laser Scanning Microscope (CLSM) Imaging exogenously generated $O_2^{-}\cdot$ and H_2O_2

The Raw 264.7 macrophages or Hela cells (2×10^4/well) were treated with **1**/MLS-**1**(5 μM) and PO1 (5 μM) for 40 min. All incubation should be conducted in DMEM medium supplemented with 10% FBS (Fetal bovine serum) and 1% penicillin at 37 ℃. After each treatment, washed 3 times with PBS buffer (10 mM, pH 7.4) to remove the residue, and subsequently fixed with 4% formaldehyde at 37 ℃ for 15 min before fluorescence observation. For **1**/MLS-**1** imaging, the emission wavelength was collected at 500–535 nm for green channel (excited at 488 nm); for PO1 imaging, the excitation wavelength was set at 543 nm, and the emission wavelength was collected at 555-700 nm for red channel. For simultaneously monitoring changes of these two probes in cells, the emission wavelength at both the green channel (500-535 nm) and red channel (555-700 nm) were collected.

To confirm that if **1**/MLS-**1** could target mitochondrial specifically, cells were treated with MitoTracker Red CMXRos (25 nM) in the final 20 min of **1**/MLS-**1** incubation. For stained mitochondria, the emission wavelength was set at 605–700 nm for red channel (excited at 594 nm). Both the green channel (500-535 nm) and red channel (605-700 nm) were collected to evaluate the mitochondrial targeting capacity of **1**/MLS-**1**.

Suppression and stimulation of exogenously generated $O_2^{-}\cdot$

Cells were pretreated with 2-ME (20 μg/ml) for 4h before further incubation with **1**/MLS-**1**, PO1 or Mitotracker red to impede the process of transferring $O_2^{-}\cdot$ to H_2O_2 by damaging the activity of SOD enzyme. Replaced with fresh medium after incubation with **1**/MLS-**1** (5 μM), PO1 (5 μM) or Mitotracker red (25 nM), followed by treating cells with PMA (5 μg/ml) for

another 30 min to stimulate exogenously generation of $O_2^{-}\cdot$. After all the treatments, the cells were washed with PBS 3 times, and subsequently fixed with 4% formaldehyde at 37 ℃ for 15 min before fluorescence observation.

Different concentrations of SWCNT-COOH (0, 5, 10, 25, 50 μg/ml) were also added to see the effect of invasion of nanotube on the exogenously generated ROS concentration in cells. Cells were pretreated with **1**/MLS-**1** (5μM) or PO1 (5μM) for 40 min, followed by incubation with SWCNT-COOH (0, 5, 10, 25, 50 μg/ml) for another 6, 12, 24 h before observation.

Cell lysis and the uptake of 1/MLS-1

The Raw 264.7 macrophages or Hela cells (1×10^4/well) were treated in a sequence with 2-ME (20 μg/ml), **1**/MLS-**1** (5 μM)), PMA (5 μg/ml) in a 96-well microtiter plate. Thereafter, clear each well with PBS for 3 times and add CelLytic^{TM} M mammalian cell lysis/extraction reagent (Sigma Aldrich，St. Louis, MO) to incubate for another 15min on a shaker.

After cell lysis, add equivalent phosphate buffer (pH 7.4, 50 mM) into each well before fluorescence detection to make the pH same. Then KO_2 (10 μM) was applied to each sample to completely reacted with **1**/MLS-**1** to evaluate the uptake under different treatments.

Cytotoxicity assay

The viability of Raw 264.7 macrophages or HeLa cells under different treatments were evaluated by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays. Cells (5×10^3/well) were seeded into a 96-well microtiter plate and grown for 24 hours at 37 ℃ in humidified environment containing 5% CO_2, followed by treatment with different concentrations (0, 1, 2.5, 5,10 μM) of **1**/MLS-**1** for another 6, 12, and 24 h. To see the toxicity of SWCNT-COOH, cells were also treated with different concentrations (0, 5, 10, 25, 50 μg/ml) of SWCNT-COOH for 6, 12 and 24 h. After each treatment, clear the wells with PBS buffer and add MTT (0.5 mg/ml) to each well and incubated for an additional 4 h. Control assay was also conducted under the same conditions except addition of MTT. All treatments are repeated 3 times.

3. Structure identification of 1

1) ^{1}H NMR spectra

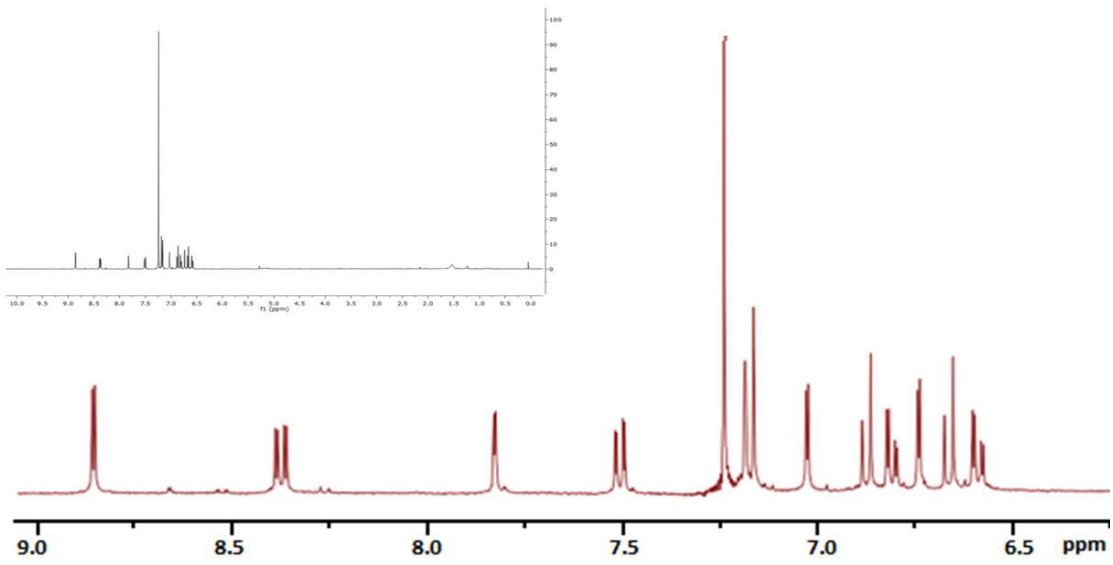

Figure S1a. ^{1}H NMR spectra of **1**.

^{1}H NMR (400 MHz, $CDCl_3$) : δ(ppm) 8.9 (d, 2H), 8.36-8.39 (dd, 2H), 7.83 (d, 1H),7.49-7.52 (dd, 2H), 7.16-7.18 (dd, 1H), 7.03 (d, 1H), 6.80-6.82 (dd, 1H), 6.73 (d, 1H), 6.66 (d, 2H), 6.57-6.60 (dd, 2H).

2) MS spectra

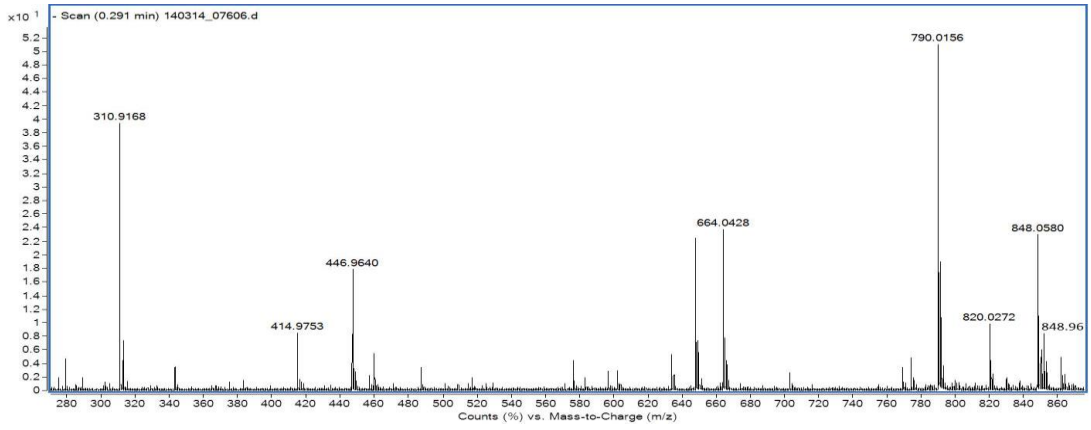

Figure S1b. LC-MS spectra of **1**.

MS: m/z calcd M^- for (M^-, $C_{33}H_{15}N_5O_{17}S_3^-$), 848.96; found, 848.96 $(M)^-$.

m/z 848.0580 $(M-H)^-$, m/z 790.0156 $(M-H-SCN)^-$, m/z 664.0428 $(M-H-C_6H_3N_2O_5-H)^-$, m/z 648.0421 $(M-H-C_6H_3N_2O_5-O-H)^-$, m/z 446.9640$(M-H-2C_6H_3N_2O_5-S-3H)^-$, m/z 414.9753 $(M-H-2C_6H_3N_2O_5S-3H)^-$, m/z 310.9168 $(M-H-SCN-2C_6H_3N_2O_6S-OH)^-$

4. Characterization of 1 in vitro system

1) Reaction rate of 1 with O_2^- ·

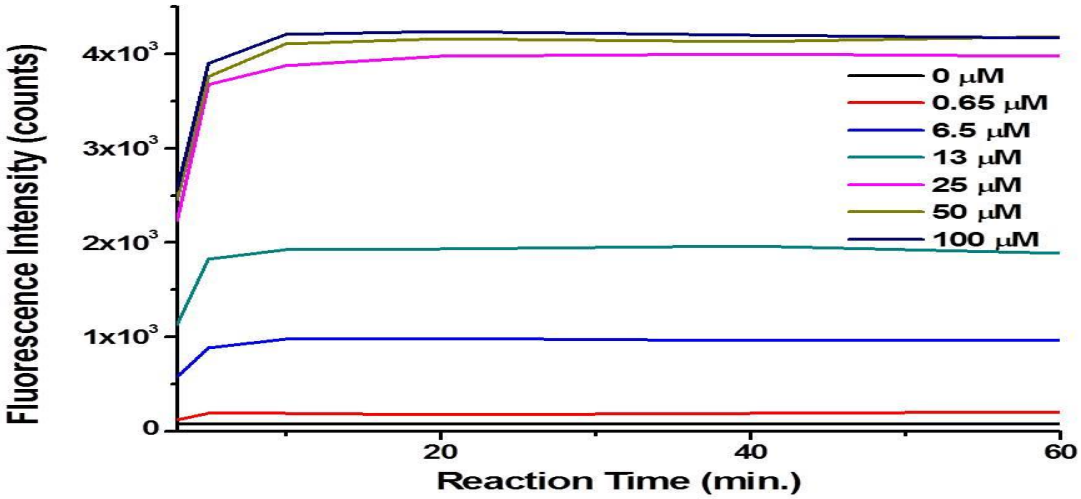

Figure S2a. Reaction rate of **1** with O_2^- : **1** (10 μM), KO_2 (0.65, 6.5, 13, 25, 50, 100 μM), phosphate buffer (50 mM, pH 7.4), t (0, 3, 5, 10, 20, 40, 60 min).

2) The sensitivity of 1 with O_2^- ·

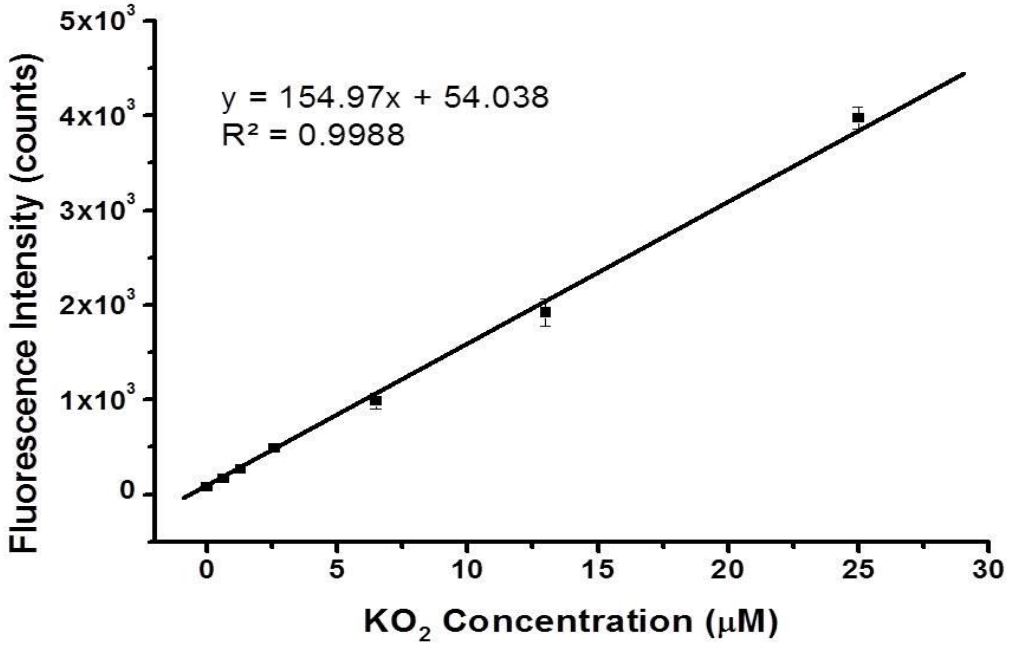

Figure S2b. The sensitivity of **1** with different concentrations of O_2^- : **1** (10 μM), KO_2 (0.65, 1.3, 2.6, 6.5, 13, 25 μM), phosphate buffer (50 mM, pH 7.4), t (10 min).

5. HPLC characterization of MLS-1

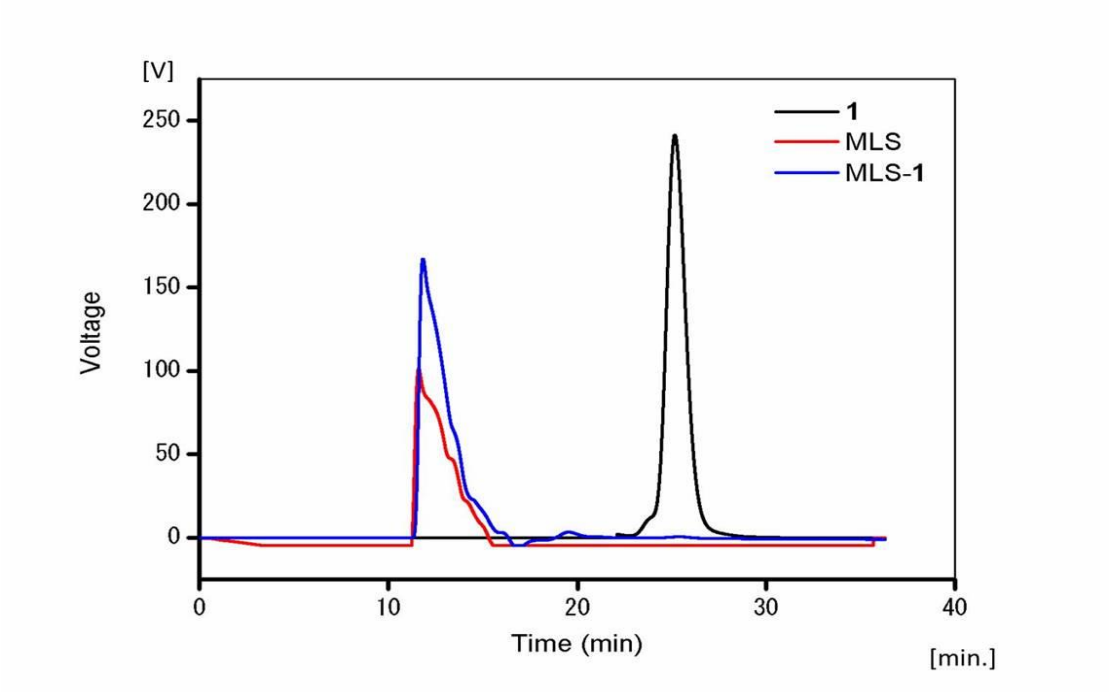

Figure S3. HPLC analysis of MLS-**1**.

6. Relative fluorescence (F/F_0) of 1 and MLS-1 after reaction with KO_2 under different pH conditions.

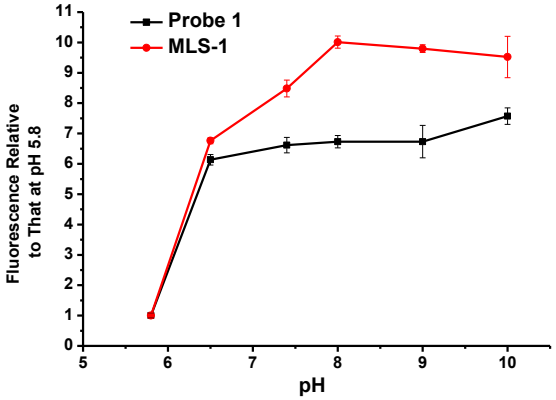

Figure S4. Five μM **1** or MLS-**1** reacted with 25 μM KO_2, and mixed with buffers at various pH values: phosphate buffer (50 mM, pH 5.8, 6.5, 7.4, 8.0), borate buffer (50 mM, pH 9.0), and bicarbonate-carbonate buffer (50 mM, pH 10.0). F_0 was the fluorescence emitted by each probe under pH 5.8, which was close to background value.

7. Cellular uptake before and after –SCN reacted with free amines.

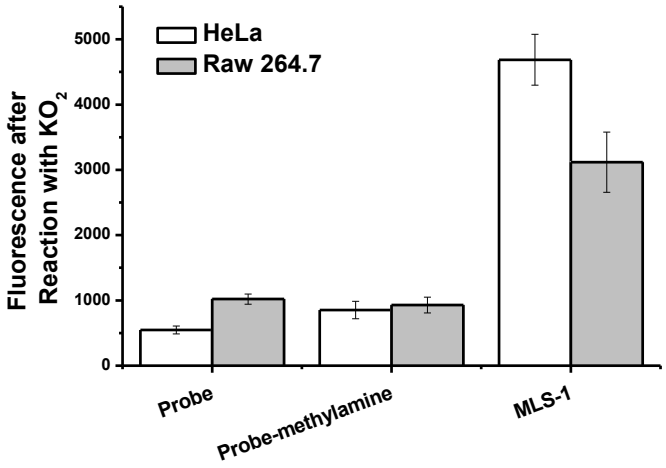

Figure S5. The concentration of **1**, **1** conjugated with methylamine, and MLS-**1** were at 5 μM, incubated with the cells for 10 min. Then the cells were lysed in 60 μl cell lysis buffer. After reaction with KO_2 at 25 μM, 60 μl phosphate buffer (50mM, pH 7.4) was supplied to maintain the neutral pH.

8. Cell viability

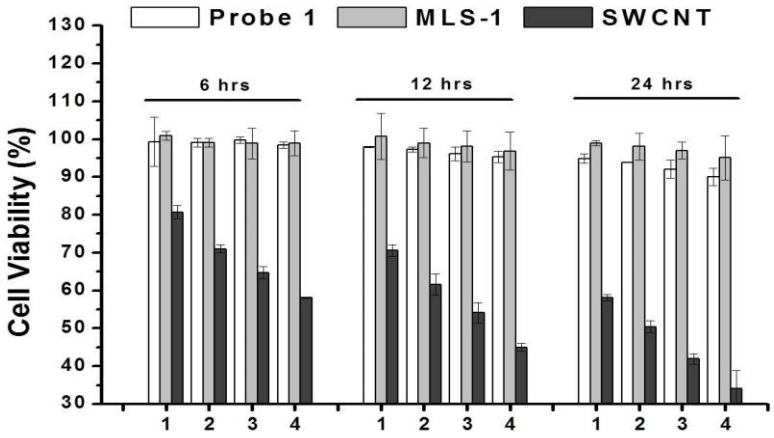

Figure S6. Cell viability values (%) estimated by MTT assay in RAW 264.7 Macrophages. **1**/MLS-**1** (1, 2.5, 5, and 10 μM, before uptake), SWCNTs (5, 10, 25, 50 μg/ml) were respectively cultured with cells for 6, 12 and 24 h.

9. CLSM imaging the intracellular location of 1

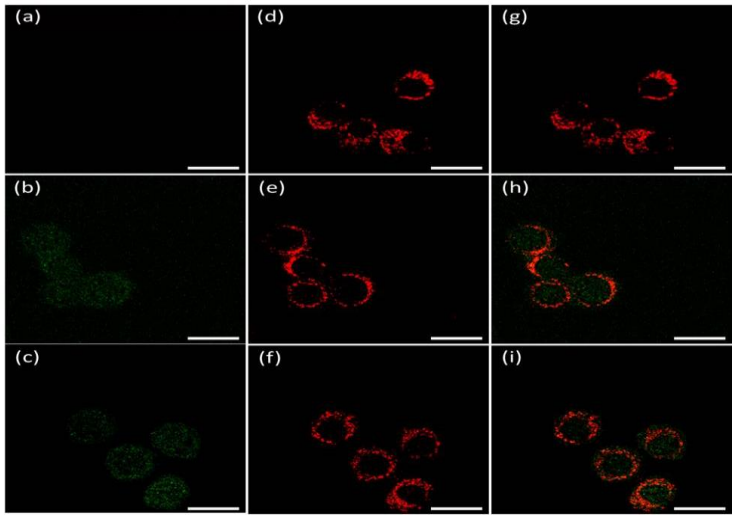

Figure S7. CLSM images of the intracellular location of **1** (5 μM, before uptake) co-stained with Mtotracker red CMXRos (25 nM) in RAW 264.7 Macrophages. The fluorescence of images were recorded at a range of (a-c) 500-535 nm (green channel, λex = 488 nm) and (d-f) 605-700 nm (red channel, λex = 594 nm) in the presence/absence of PMA (stimuli of O_2^{-} ·)/ 2-ME(inhibitor of SOD). (g-i) The overlay image of green and red channel. Scale bar = 20 μm

10 Monitoring intracellular O_2^{-} ·and H_2O_2 simultaneously with MLS-1 and PO1.

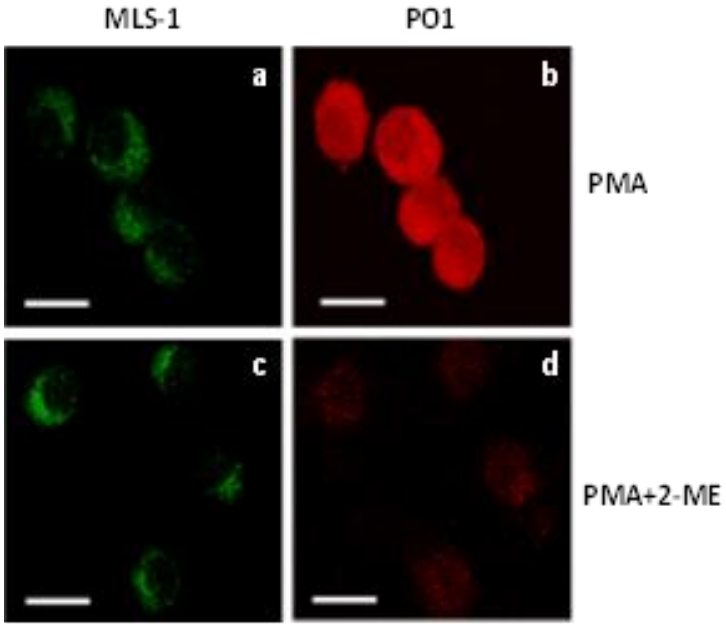

Figure S8. Confocal fluorescence images of MLS-1 co-stained with PO1 in RAW 264.7 Macrophages. The green (λex = 488 nm; λem = 500-535 nm) (a and c) and red (λex = 543 nm; λem = 555-700 nm (b and d) channels were collected in cells stimulated by PMA (a-b) alone or in combination with 2-ME (c-d). Scale bar = 20 μm.

11. Monitoring intracellular O_2^- ·concentration with invasion of SWCNTs

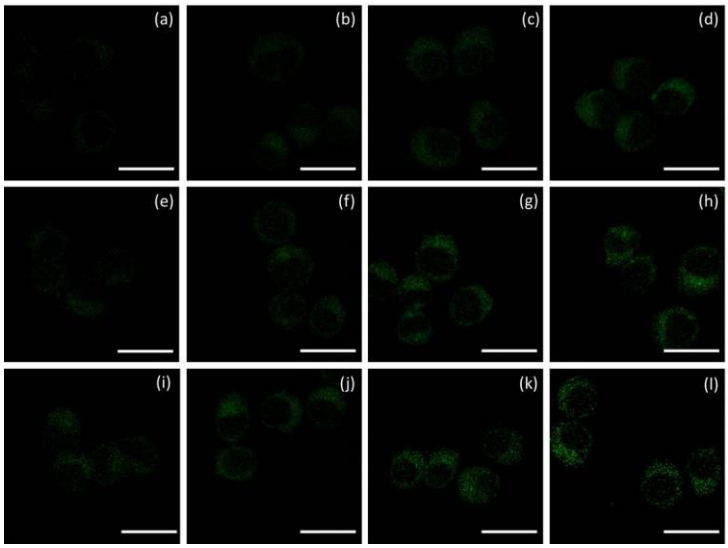

Figure S9. CLSM imaging changes of intracellular O_2^- ·concentration with invasion of SWCNTs in RAW 264.7 Macrophages. Pre-loaded MLS-**1**(5 μM, before uptake) cells were further incubated with different concentrations of SWCNTs (5，10, 25， 50 μg/ml) for (a-d) 6 hrs, (e-h) 12 hrs, and (i-l) 24 hrs. Green channel (500-535 nm). λex = 488 nm; Scale bar = 20 μm

12. Hela cells

1) Cell viability

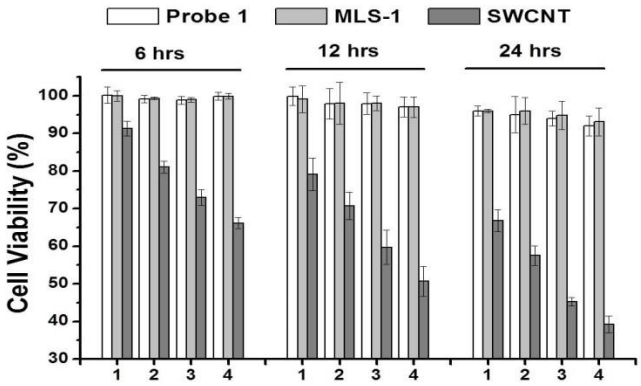

Figure S10a. Cell viability values (%) estimated by MTT assay in Hela cells. **1**/MLS-**1** (5 μM, before uptake), SWCNTs (5, 10, 25, 50 μg/ml) were respectively cultured with cells for 6, 12 and 24 h.

2) CLSM imaging the intracellular location of MLS-1

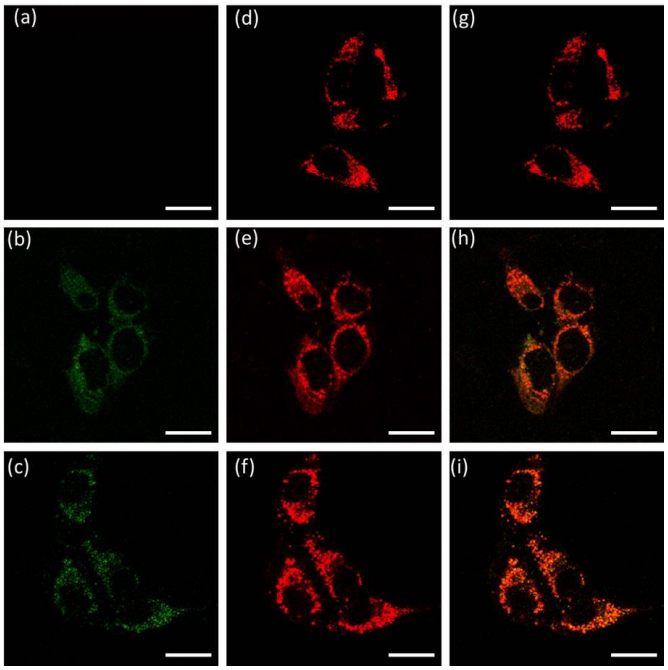

Figure S10b. CLSM images of the intracellular location of MLS-**1** (5 μM, before uptake) co-stained with Mtotracker red CMXRos (25 nM) in Hela cells. The fluorescence of images were recorded at a range of (a-c) 500-535 nm (green channel, λex = 488 nm) and (d-f) 605-700 nm (red channel, λex = 594 nm) in the presence/absence of PMA (stimuli of O_2^{-} ·)/ 2-ME(inhibitor of SOD). (g-i) The overlay image of green and red channel. Scale bar = 20 μm

3) Simultaneous monitoring intracellular generation of O_2^{-} and H_2O_2

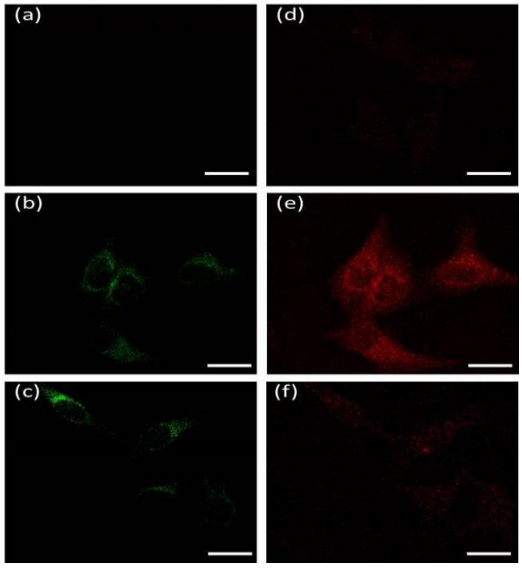

Figure S10c. Simultaneous CLSM imaging intracellular generation of O_2^{-} and H_2O_2 in Hela cells. MLS-**1** (5 μM, before uptake), PO1 (5 uM, before uptake). The fluorescence of images were collected at a range of (a-c) 500-535 nm (green channel, λex = 488 nm) and (e-f) 555-700 nm (red channel, λex = 543 nm) in the presence of PMA (stimuli of O_2^{-} ·)/ 2-ME (inhibitor of SOD). Scale bar = 20 μm