Supporting Information

Mitochondria-targeted fluorescent probe for visualization of exogenous and endogenous of methylglyoxal in cancer cells

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1. Materials and instrumentation

Starting materials and reagents were commercially available and used without further purification. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MitoTracker® Green FM, LysoTracker Green DND-26 and ER-Tracker Green (BODIPY® FL Glibenclamide) were purchased from Beyotime Institute of Biotechnology. NMR spectra were recorded on a Bruker AVANCE III HD 400MHz spectrometer. Chemical shifts (d values) were reported in ppm down field from internal Me_4Si (^1H and ^{13}C NMR). High resolution mass spectra (HRMS) were acquired on an Agilent 6510 Q-TOF LC/MS instrument (Agilent Technologies, Palo Alto, CA) equipped with an electrospray ionization (ESI) source. Melting points were recorded on a RY-2 apparatus (Tianjin, China). Fluorescence spectra were recorded on a Hitachi F-4600 spectrofluorophotometer with a 1 cm standard quartz cell. The pH measurements were carried out on a Mettler Toledo SevenExcellence pH meter. The absorbance for MTT analysis was recorded on a microplate reader (PL-9602). Cells imaging was performed with Olympus FV1000-IX81 confocal microscope. All images were analyzed with Olympus FV1000-ASW and image J.

2. Experiment

*2.1 Preparation of the probe **Z1***

Z1 has been synthesized according to the published procedure [S1].

*2.2 Preparation of the probe **Z2***

To a 50 mL flask, was charged *N*-methyl-2,3,3'-trimethylindolium iodide (602 mg, 2 mmol), 4-[(1-methylethyl) amino]-3-nitrobenzaldehyde (458 mg, 2.2 mmol) and anhydrous ethanol (15 mL). The reaction mixture was washed with nitrogen flow for 30 min to remove oxygen, and then stirred at 90 ^oC for 24 h. After cooling to room temperature, KBF_4 (504 mg, 4 mmol) was added in one portion to the reaction mixture. The suspension was refluxed for another 2 h in dark. The suspension was filtered through a short celite column and the filtrate was condensed to dryness under reduced pressure. The residue was recrystallized from acetonitrile (10 mL). **Z2** was obtained as an orange powder (817 mg) in 97% yield; mp 139-141 ^oC; HRMS: m/z $[M\text{-}BF_4^-]^+$ = 364.2044; Calcd: 364.2020; ^1H NMR (400 MHz, $CDCl_3$) (δ, ppm) 8.65

(d, J = 6.8 Hz, 1H), 8.52 (d, J = 9.2 Hz, 1H), 8.48 (s, 1H), 8.05 (d, J = 15.6 Hz, 1H), 7.54 (d, J = 15.2 Hz, 1H),7.53 (d, J = 4.4 Hz, 3H), 7.15 (d, J = 8.8 Hz, 1H), 4.17 (s, 1H), 4.04-3.98 (q, J = 6.4 Hz, 1H), 1.81 (s, 6H), 1.38(d, J = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) (δ, ppm) 181.68, 153.44, 148.11, 142.98, 141.91, 135.97, 133.81, 131.55, 129.98, 129.74, 122.72, 122.09, 116.80, 114.61, 109.89, 52.31, 45.32, 34.70, 27.32, 22.95.

*2.3 Preparation of the probe **Hcy-OPD***

To a solution of **Z2** (200 mg, 0.55 mmol) in ethanol, $SnCl_2 \cdot 2H_2O$ (1.5 g, 6.6 mmol) was added and the mixture was refluxed overnight for 12 h. The reaction was purified by silica gel column chromatography (SiO_2, CH_2Cl_2/MeOH, v/v, 100/3) to obtain a green solid **Hcy-OPD** in 40% yield (73 mg); m.p. 209-210 ^{o}C; HRMS: m/z $[M\text{-}BF_4^-]^+$ = 334.2286; Calcd: 334.2278; ^{1}H NMR (400 MHz, $CDCl_3$) (δ, ppm) 8.14 (d, J = 15.2 Hz, 1H), 7.73 (d, J = 7.2 Hz, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.51 (t, J = 7.2 Hz, 2H), 7.42 (t, J = 7.4 Hz, 1H), 7.34 (s, 1H), 6.97 (d, J = 15.2 Hz, 1H), 6.67 (d, J = 8.8 Hz, 1H), 3.89 (s, 3H), 1.72 (s, 6H), 1.26 (d, J = 6.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) (δ, ppm): 177.81, 154.33, 147.16, 142.36, 141.75, 135.47, 130.23, 129.66, 129.33, 127.24, 124.19, 122.39, 112.45, 109.74, 103.53, 50.56, 45.08, 33.07, 27.85, 22.54.

3. General procedure for the spectrum measurement

The stock solution of the probe **Hcy-OPD** was prepared at 5 mM in DMF. The test solution was in 10 mM PBS buffer (pH 7.4). The resulting solution was shaken well and incubated for 1 h at 37 ^{o}C before recording the spectra. Unless otherwise noted, for all the measurements, the excitation wavelength was 570 nm, the excitation slit width was 5 nm, and emission slit width was 10 nm.

4. Detection limit

The fluorescence emission spectra of **Hcy-OPD** (5 μM) were measured by eleven times, thus the standard deviation of blank sample was obtained. Then, the fluorescence intensity at 658 nm was plotted as the concentration of methyglyoxal based on fluorescence titration experiment. The detection limit was calculated by using:

$$LOD = 3\sigma/k$$

where “σ” is the standard deviation of blank sample, *k* is the slope between the fluorescence intensity ($F_{658\ nm}$) *versus* the concentration of methyglyoxal.

5. Competition experiments

The stock solution of the probe **Hcy-OPD** was prepared at 5 mM in DMF. In a 5 mL tube, 3 mL of 10 mM PBS buffer (pH 7.4) and 3 μL of the stock solution of **Hcy-OPD** were mixed, followed by addition of an appropriate volume of methylglyoxal solution. Then, the various species were added to each tube, after well mixed, the solutions were allowed to stand at 37 °C for 1 h before recording the spectra. In the meantime, a blank solution containing no methylglyoxal was prepared and measured under the same conditions for comparison. λ_{ex} = 570 nm, slit = 5 nm, 10 nm.

6. Cells culture

Hela cells were grown in RPMI 1640 supplemented with 10% FBS (fetal bovine serum) under an atmosphere of 5% CO_2 at 37 ^{o}C, and then divided into 2 groups, respectively. The cells were seeded at a density of 1 × 10^6 cells per mL in confocal dishes for imaging and allowed to adhere for 24 h.

7. Confocal imaging:

Exogenous imaging: the Hela cells were treated with 0.5 μM probe in culture medium for 1 h at 37 ^{o}C and washed three times with PBS. For the control experiment, HeLa cells was treated 0.5 μM probe for 1 h at 37 ^{o}C and 0.5 mM MGO was added to the cells, and then incubated for another 1 h at 37 ^{o}C. Then, the medium was removed and washed three times with PBS to remove the excess probe and residual MGO.

Endogenous imaging: For the fluorescence imaging of the endogenous MGO in living HeLa cells, the control experiment, HeLa cells were incubated with 5 mM *N*-acetylcysteine (NAC) or aminoguanidine (AG) for 1 h. For the experimental group, HeLa cells pretreated with NAC or AG then was cultured with 1 μM **Hcy-OPD** for another 1 h.

Colocalization imaging: Probe dissolved in DMSO were added to the cells medium (500 μL) at a final concentrations (0.5 μM). After incubating for 30 min, free

probe was removed by gentle rinsing with phosphate buffered saline (PBS, pH 7.4) three times. To observe the subcellular distributions of the probe, the cells were treated with a staining probe, Lyso-Tracker Green DND-26 (200 nM), ER-Tracker Green (200 nM) or Mito-Tracker Green (200 nM) for additional 30 min at 37 ℃. And then washed with PBS three times. Lyso-Tracker Green DND-26, ER-Tracker Green and Mito-Tracker Green were excited at 488 nm and green emissions were collected between 509 nm and 535 nm; probe was excited at 559 nm and their red emissions were collected between 585 nm and 685 nm.

8. Cell viability assay

MTT assay: To examine the toxicity of the probe in living cells, HeLa cells were incubated with different concentrations (0.1μM, 1 μM, 5 μM, 10 μM, 20 μM) of the probe in DMEM medium supplemented with 10% fetal bovine serum (FBS) and allowed to grow for 24 h at 37 °C with 5% CO_2. Repeat 5 times at each concentration. After removal of the medium, cells in each well were treated with 10 μL MTT solution (5 mg/mL), and incubated for another 4 h. Before measurements, cells were treated with 100 μL DMSO at 37 ºC for 2 h, and then the OD490 data for each well were recorded on PL-9602. The viability was calculated by using the formula: (experiment group - background)/(blank group - background) × 100%.

LDH assay: Adherent HeLa cells were incubated with different concentrations (0.1μM, 1 μM, 5 μM, 10 μM, 20 μM) of the probe in DMEM medium supplemented with 10% fetal bovine serum (FBS) and allowed to grow for 24 h at 37 °C with 5% CO_2. Repeat 5 times at each concentration. The culture plate was taken out 1 h before the end point of action, and the 10% LDH releasing reagent was provided in a control group containing only the medium, then continued to be incubated to the end point. After incubated 24 h, 120 μL supernatant in each well were placed in another 96-well plate, adding 60 μL LDH working solution and incubated for another 30 min at room temperature. The OD490 data for each well were recorded on PL-9602. The LDH leakge (cell death ratio) was calculated by using the formula: (experiment group - background)/(blank group - background) × 100%.

Table S1 The photophysical properties of **Hcy-OPD** and **Hcy-OPD-MGO**.

	λ_{Abs} (nm)	λ_{Em} (nm)	ε (M^{-1} cm^{-1})	Φ
Hcy-OPD	545	626	3.87× 10^4	0.011
Hcy-OPD-MGO	536	658	322	0.014

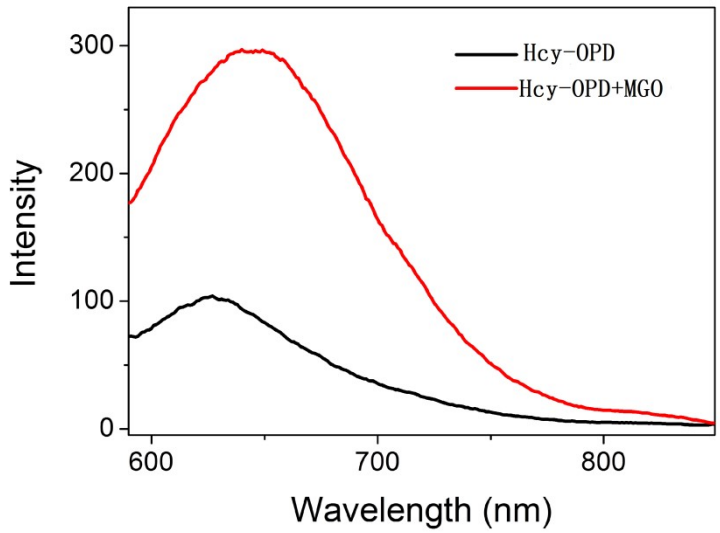

Figure S1. Fluorescence spectra of probe **Hcy-OPD** (5 μM) before and after adding MGO (500 equiv.) at 37 °C for 1 h in PBS buffer (10 mM, pH 7.4). λ_{ex} = 570 nm, slit = 5 nm, 10 nm.

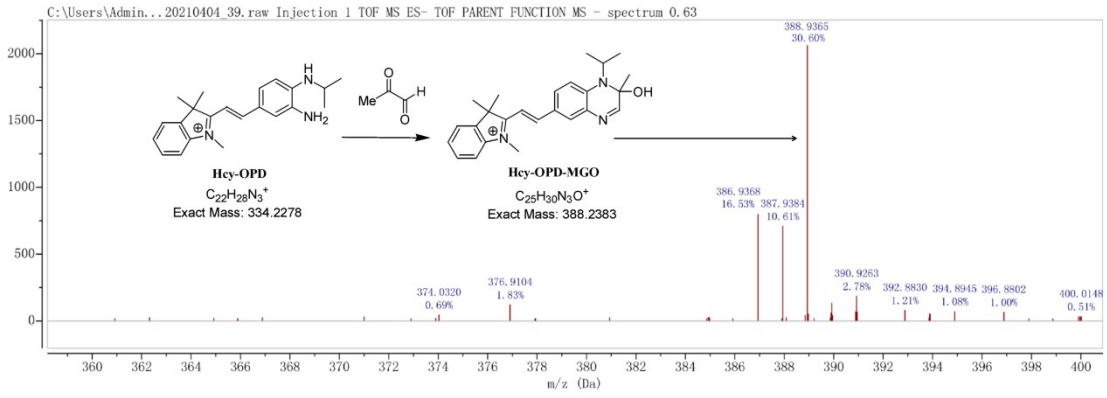

Figure S2. HRMS (LC/MS) spectrum of **Hcy-OPD** in the presence of MGO in PBS buffer (10 mM, pH 7.4). The m/z peak of 388.9365 can be assigned to the reaction product of the probe **Hcy-OPD** in the presence of MGO.

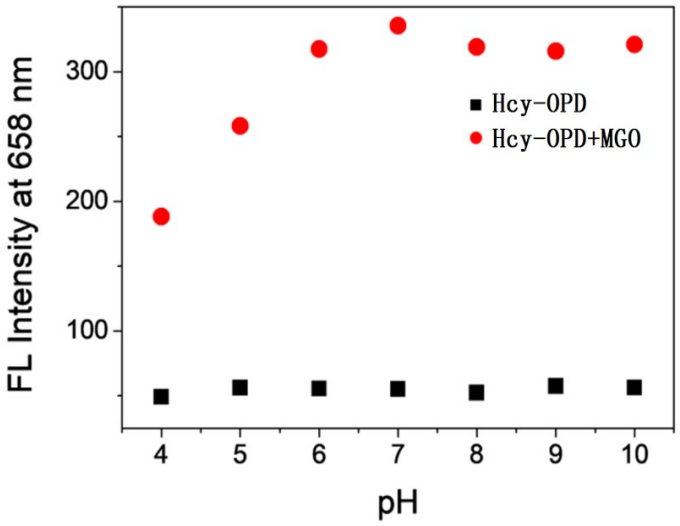

Figure S3. Fluorescence intensity changes of **Hcy-OPD** (5 μM) at 658 nm in the presence and absence of 500 equiv. MGO at different pH. λ_{ex} = 570 nm; slit = 5 nm, 10 nm.

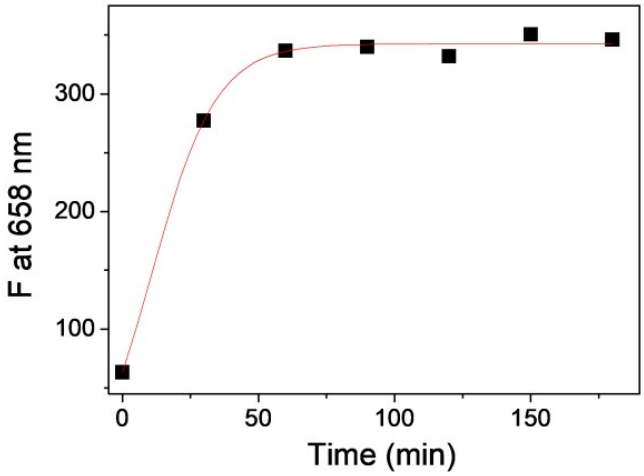

Figure S4. Time-dependent fluorescence intensity of **Hcy-OPD** (5 μM) at 658 nm upon the addition of 500 equiv. MGO at 37 °C.

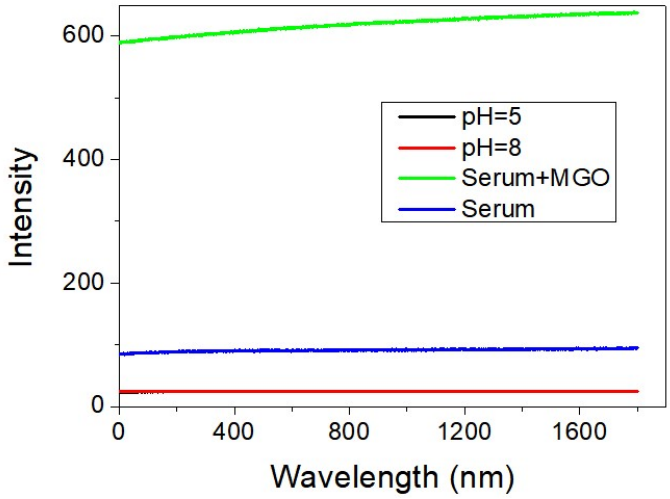

Figure S5. Time-dependent fluorescence intensity of **Hcy-OPD** (5 μM) at 658 nm in in acidic/basic PBS buffers solution (pH = 5 and pH = 8), and serum.

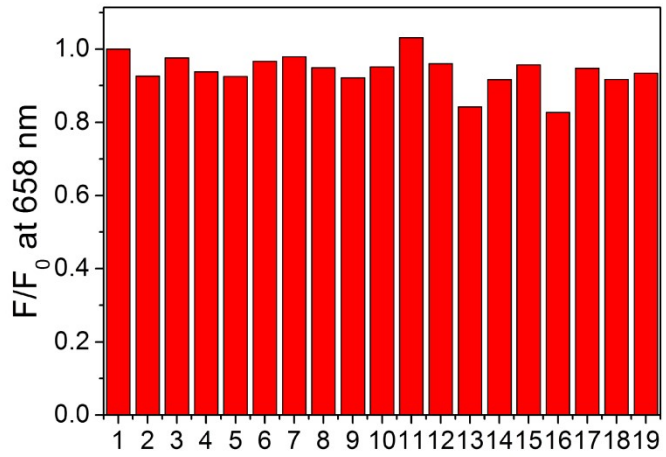

Figure S6. Fluorescence response of **Hcy-OPD** upon reaction with various species. 1: MGO (5 μM); 2: MGO (5 μM) + arginine (50 μM); 3: MGO (5 μM) + glucose (50 μM); 4: MGO (5 μM) + histidine (50 μM); 5: MGO (5 μM) + lysine (50 μM); 6: MGO (5 μM) + alanine (50 μM); 7: MGO (5 μM) + glutamic acid (50 μM); 8: MGO (5 μM) + Cys (50 μM); 9: MGO (5 μM) + Hcy (50 μM); 10: MGO (5 μM) + GSH (50 μM); 11: MGO (5 μM) + formaldehyde (5 mM); 12: MGO (5 μM) + glyoxal (5 mM); 13: MGO (5 μM) + acetaldehyde (5 mM); 14: MGO (5 μM) + anisaldehyde (5 mM); 15: MGO (5 μM) + H_2O_2 (50 μM); 16: MGO (5 μM) + NO (50 μM); 17: MGO (5 μM) + Ca^{2+} (50 μM); 18: MGO (5 μM) + Cu^{2+} (50 μM); 19: MGO (5 μM) + K^+ (50 μM).

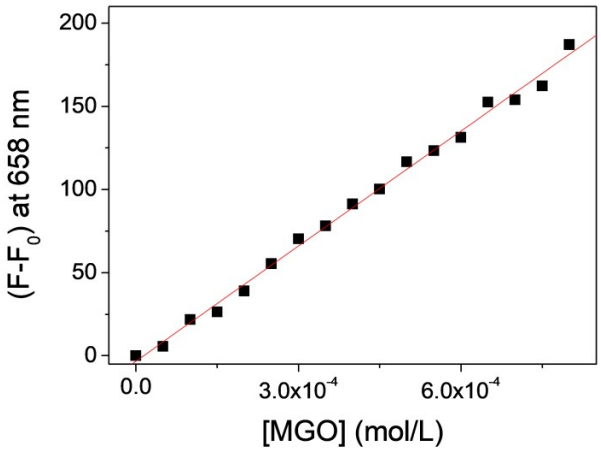

Figure S7. Fluorescence intensity changes at 658 nm of **Hcy-OPD** (5 μM) in the presence of different concentrations of MGO. The limit of detection (LOD) can be calculated with the equation [S2], LOD = 3σ/*k*, where "*k*" is the calibration sensitivity of the fluorescence intensity changes ($\Delta F=F - F_0$) *versus* [MGO], and "σ" is the standard deviation of the blank signal (F_0) obtained without MGO ($\sigma_{\mathbf{Hcy\text{-}OPD}} = 0.017$). LOD for MGO was calculated to be 2.2×10^{-7} mol/L under the testing conditions.

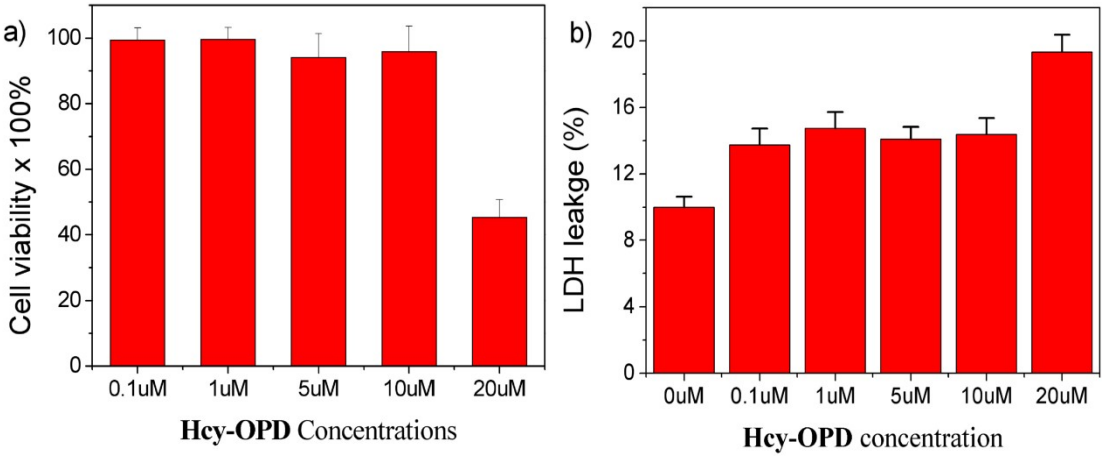

Figure S8. a) Cell viability and b) LDH leakge for HeLa cells in the presence of the probe at varied concentrations for 24 h.

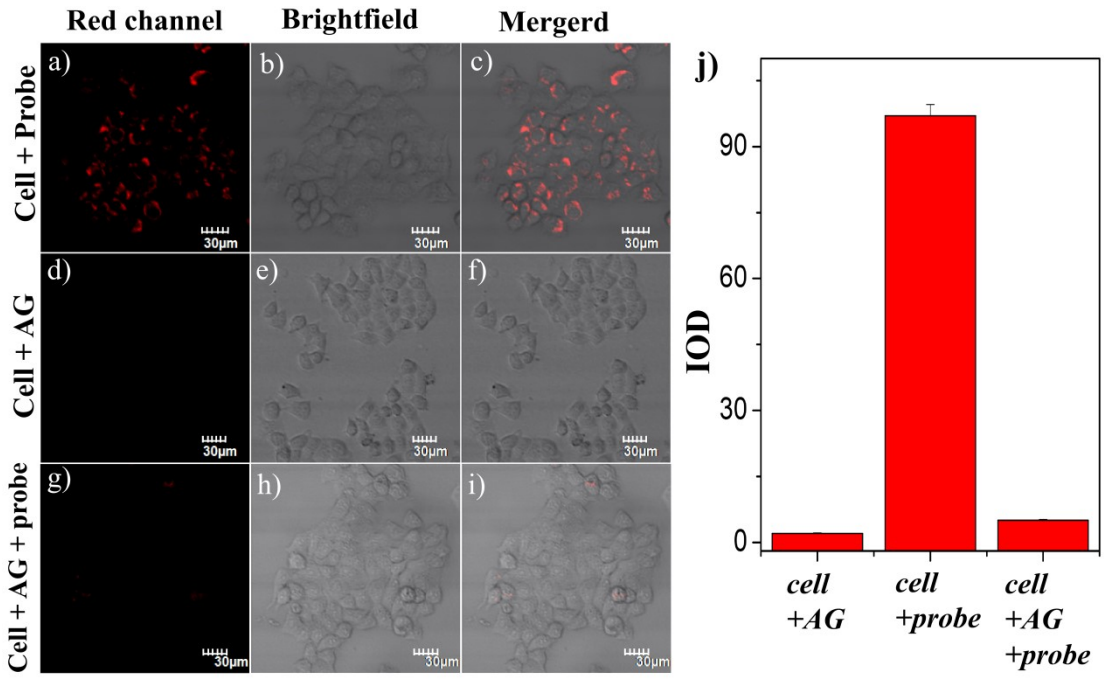

Figure S9. Confocal fluorescence images for endogenous MGO. (a-c) Cells were incubated with with **Hcy-OPD** (1 μM) for 1 h. (d-f) HeLa cells were incubated with **AG** (5 mM) for 1 h. (g-i) Cells were pre-incubated with AG (5 mM) for 1 h and then with **Hcy-OPD** (1 μM) for another 1 h. j) Mean fluorescence intensity in red channel by the Image J, the results were presented as means ± SE with replicates n = 3. λ_{ex} = 559 nm, collected at 600-700 nm.

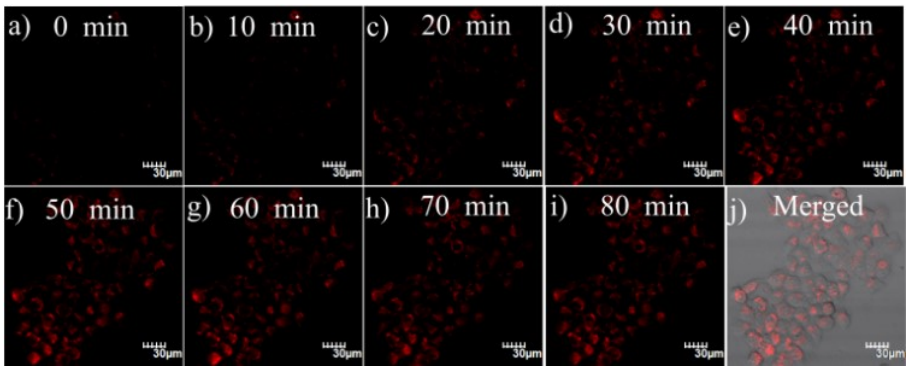

Figure S10. Time-dependent confocal fluorescence imaging of the endogenous MGO in living HeLa cells. HeLa cells were treated with the probe **Hcy-OPD** (1 μM), and the images were taken every 10 min for a total of 80 min. The incubation time for the probe with the cells is shown on the top of each panel. λ_{ex} = 559 nm, collected at 600-700 nm. Scale bar: 30 μm.

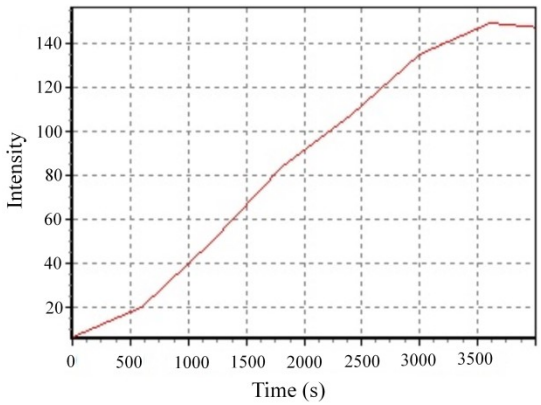

Figure S11. Time-dependent confocal fluorescence imaging of the endogenous MGO in living HeLa cells.

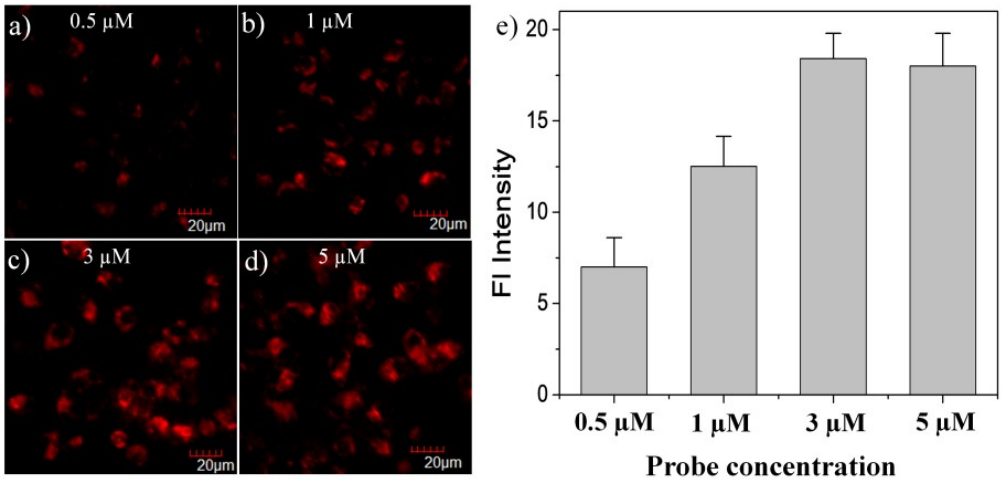

Figure S12. (a-d) Confocal fluorescence images for monitoring exogenous MGO. HeLa cells were incubated with different concentrations of **Hcy-OPD** for 1 h. λ_{ex} = 559 nm, collected at 600-700 nm. (e) Relative fluorescence intensity output of group (a-d). Scale bar: 20 μm.

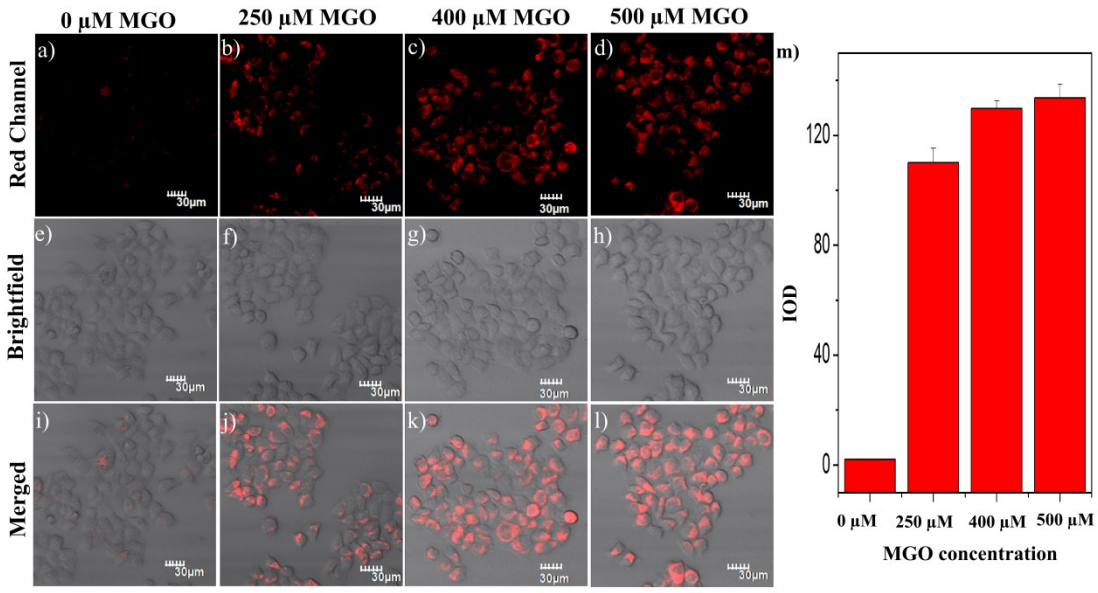

Figure S13. Confocal fluorescence images for monitoring intracellular MGO. HeLa cells were incubated first with NAC (5 mM), **Hcy-OPD** (0.5 μM) for 1 h, respectively; then with different levels of MGO (0 μM, 250 μM, 400 μM, and 500 μM) for 1 h.

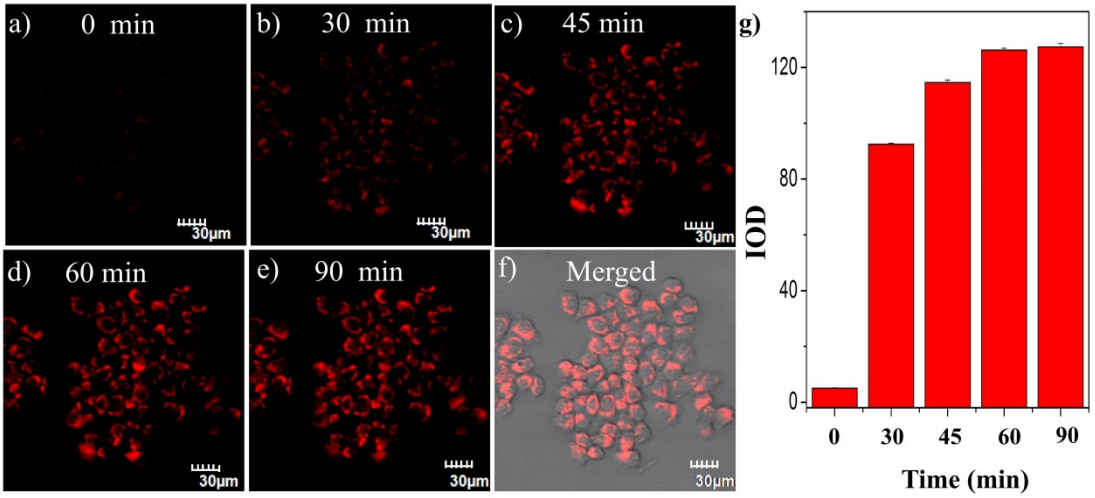

Figure S14. Time-dependent confocal fluorescence imaging of the exogenous MGO in living HeLa cells. Cells were pre-incubated with NAC (5 mM), **Hcy-OPD** (1 μM) for 1 h, and respectively; then adding 500 μM MGO. λ_{ex} = 559 nm, λ_{em} = 600-700 nm.

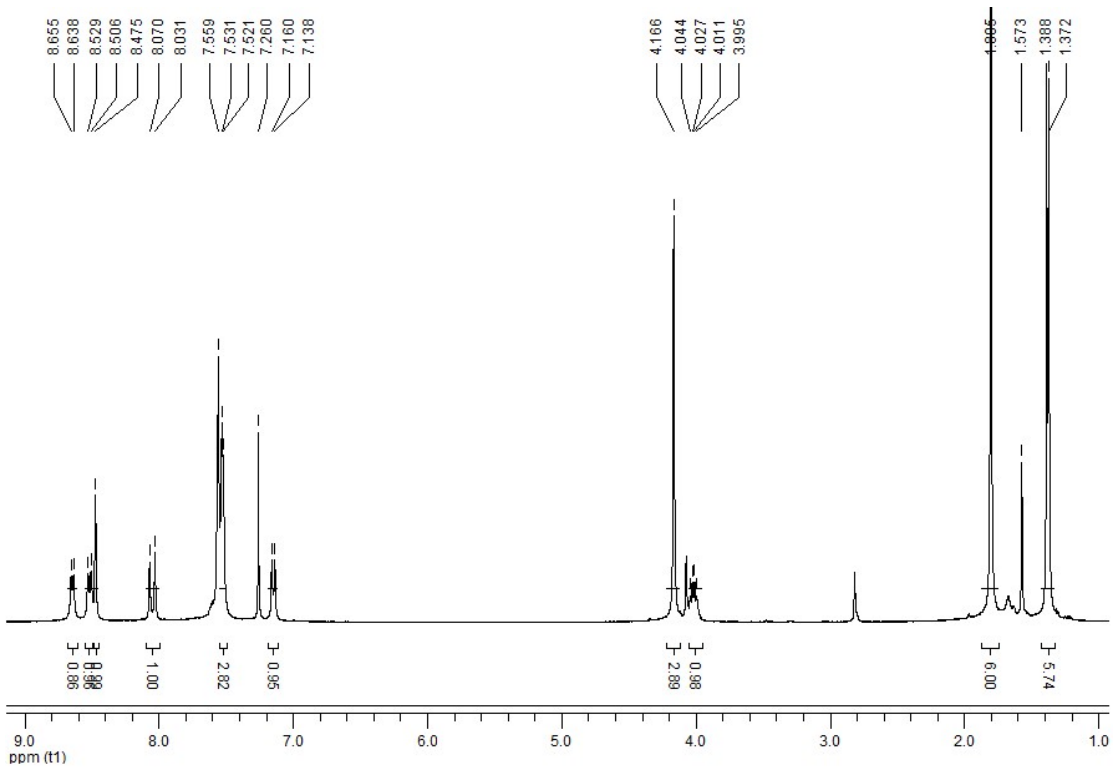

Figure S15. ^{1}H NMR of **Z2** (400 MHz, $CDCl_3$).

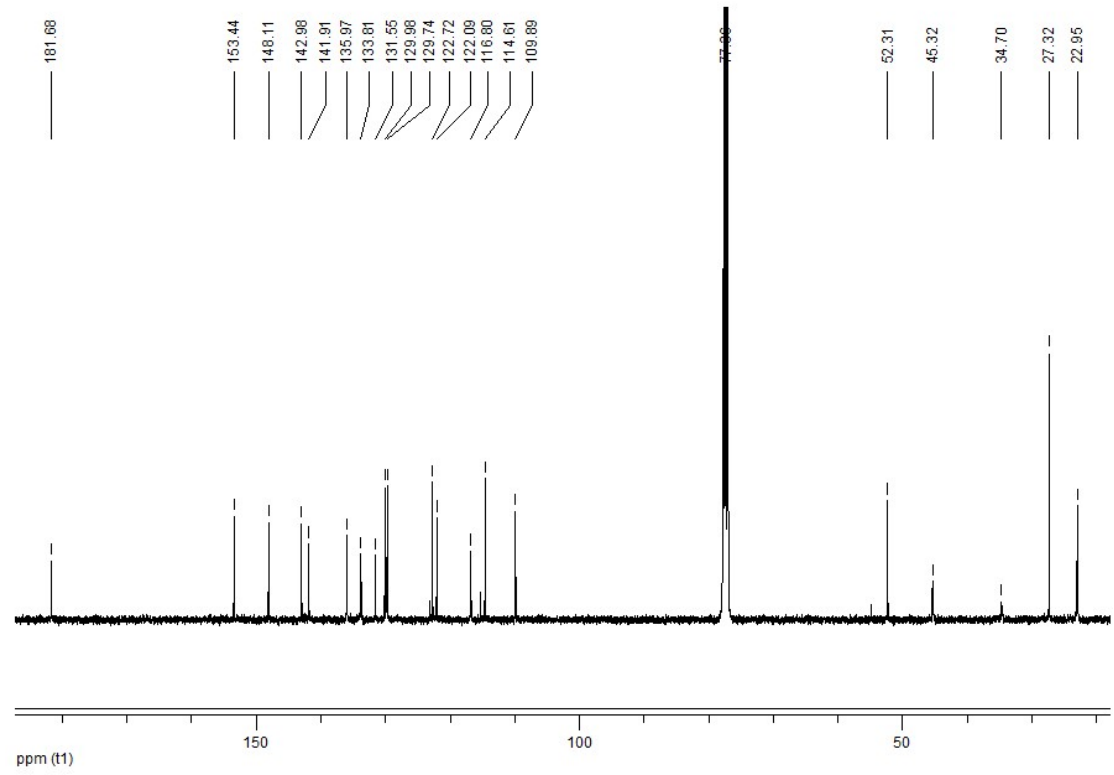

Figure S16. ^{13}C NMR of **Z2** (100 MHz, $CDCl_3$).

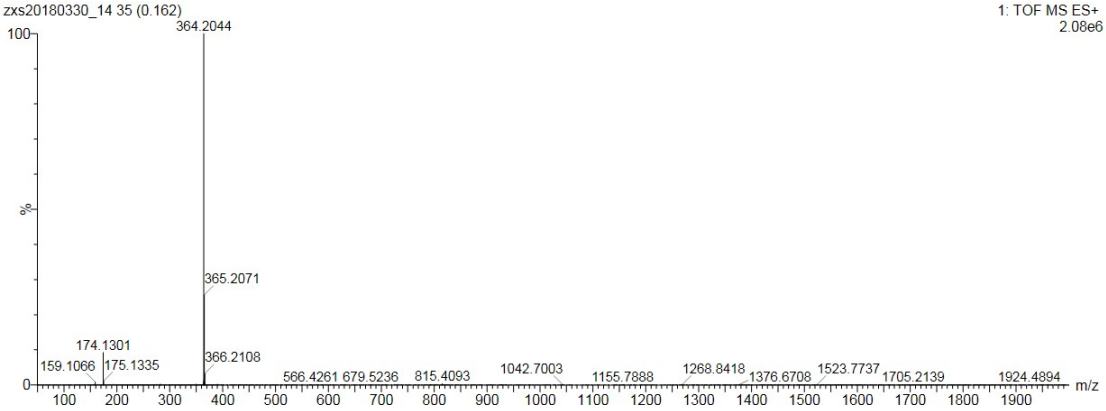

Figure S17. HRMS (LC/MS) spectra of **Z2**. The peak at m/z = 364.2044 was assigned to the mass of [**Z2**]$^{+}$.

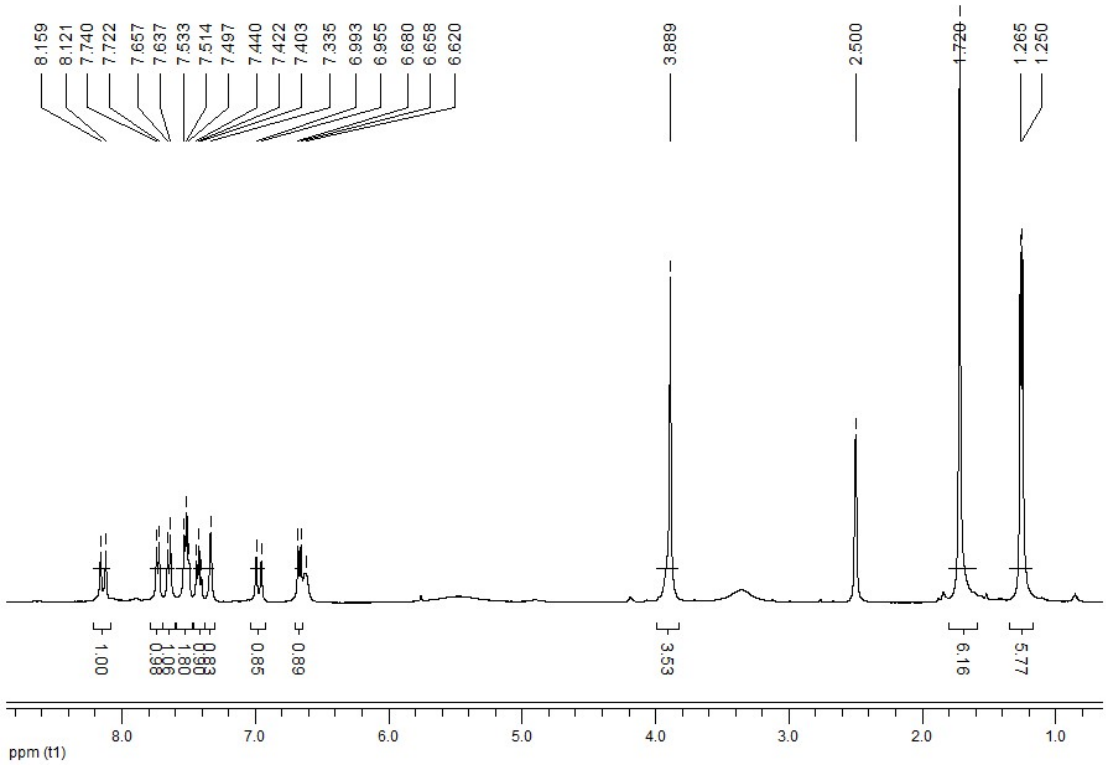

Figure S18. ^{1}H NMR of **Hcy-OPD** (400 MHz, $CDCl_3$).

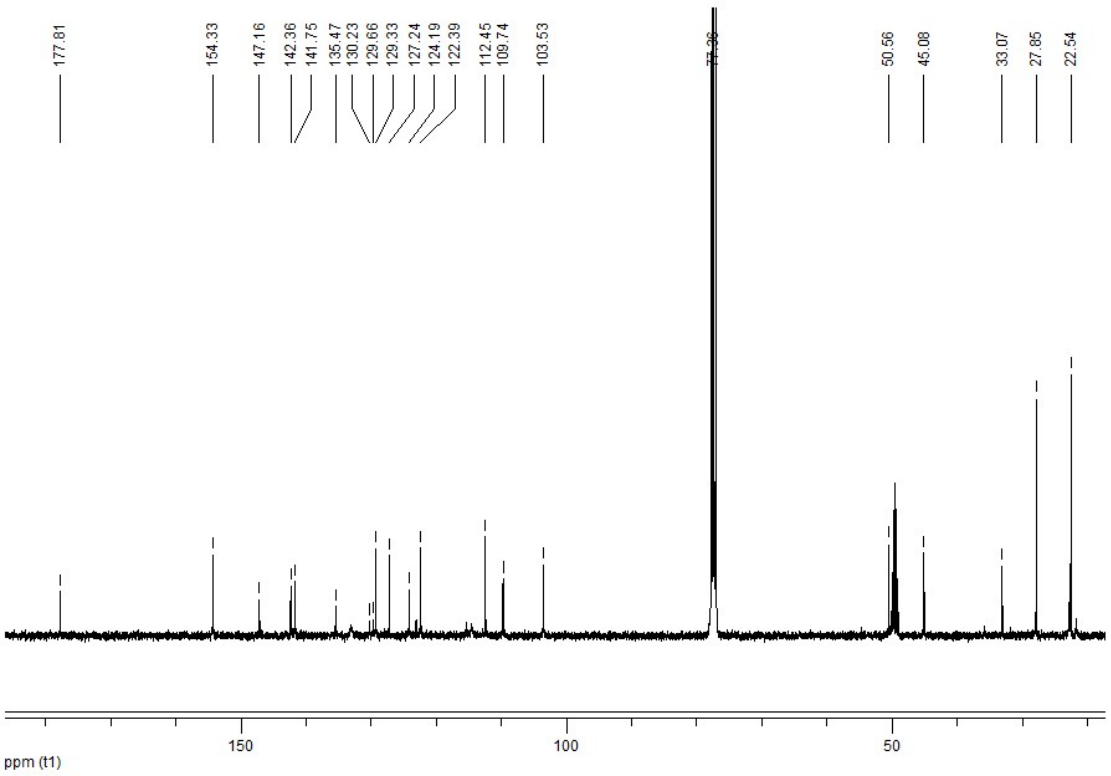

Figure S19. ^{13}C NMR of **Hcy-OPD** (100 MHz, $CDCl_3$).

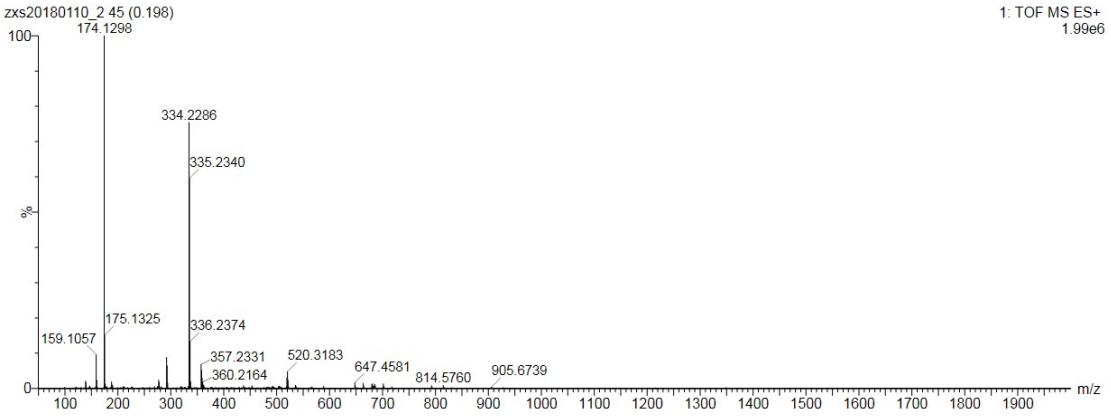

Figure S20. HRMS (LC/MS) spectra of **Hcy-OPD**. The peak at m/z = 334.2286 was assigned to the mass of [**Hcy-OPD**]$^+$.

Reference

[S1] M. Selvaraju, S. Dhole, C.M. Sun, Three component divergent reactions: base-controlled amphiphilic synthesis of benzimidazole-linked thiazetidines and fused

thiadiazines, *J. Org. Chem.*, 2016, **81**, 8867-8875.

[S2] B. Zhang, X. Yang, R. Zhang, Y. Liu, X. Ren, M. Xian, Y. Ye, Y. Zhao, *Anal. Chem.*, 2017, **89**, 10384-10390.