Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Neutral water-soluble mitochondria-targeting polymer

Yu Geng, [a] ‡ *Yin Zhong,* [a] ‡ *Quan Zhou,* [a] *Siqin Chen,* [a] *Ying Piao,* [a] *Wei Yin,* [b] *Hua Lu,* [c] *and Youqing Shen**[a]

‡ These authors contributed equally to this work.

Experimental Section

Materials

All materials other than indicated were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Dichloromethane (CH_2Cl_2, DCM), tetrahydrofuran (THF) and triethylamine (TEA) were distilled over calcium hydride (CaH_2) or treated with 4Å molecular sieve. 3-Chloroperoxybenzoic acid (mCPBA), trifluoroacetic acid (TFA), 2,2'- bipyridyine, 4-dimethylaminopyridine (DMAP), *N*-hydroxysuccinimide (NHS), and *tert*-butyl methacrylate were purchased from Aladdin (Shanghai, China). 2-(*N,N*-Diethylamino)ethyl methacrylate (DEA) was purchased from Sigma-Aldrich (Shanghai, China). Cy5.5-NHS was purchased from Lumiprobe Corporation (Shanghai, China). Hoechst 33342(Cat. No. R37605) and Mito-Tracker Green (Cat. No. M7514) were purchased from Thermo Fisher Scientific (Shanghai, China). JC-1 mitochondrial membrane potential assay kits were obtained from Beyotime Biotechnology (Haimen, China). α-Methoxypoly(ethylene glycol)-ω-amine with a molecular weight of 20kDa (mPEG-NH_2-20K) was purchased from Yare Biotechnology (Shanghai, China). It was reacted with Cy5.5-NHS and then purified by dialysis to produce $^{Cy5.5}$PEG.

Instruments

^{1}H NMR spectra were obtained on a 400 MHz Varian Gemini NMR spectrometer in deuterated solvents as noted. Gel permeation chromatography (GPC) was performed on a Wyatt GPC/SEC-MALS system (Wyatt Technology Corporation, Santa Barbara, USA) equipped with a DAWN® HELEOS® II 18-angle static light scattering detector and an Optilab® T-rEX™ refractive index detector, and three columns in series (MZ GPC-PRECOLUMN 50 × 8.0 mm MZ-Gel SDplus 100Å 10 μm, a 300 × 8.0 mm MZ-Gel SDplus 10^4Å 10 μm and 300 × 8.0 mm MZ-Gel SDplus 100Å 10 μm) at 50 ^{0}C using DMF containing 50 mM LiBr as eluent at a flow rate of 0.80 mL/min. Data were recorded and processed with ASTRA v6.0 software (Wyatt Technology Corporation, Santa Barbara, USA). Samples were dissolved in DMF at a concentration of 10 mg/mL and filtered with 0.22 μm filters.

Synthesis of poly[2-(*N*,*N*-diethylamino)ethyl methacrylate] (PDEA)

2,2'- Bipyridyine (0.071 g, 0.45 mmol) and CuBr (0.033 g, 0.23 mmol) were added into a schlenk flask. The schlenk flask was degassed by five cycles of vacuum and nitrogen purging for 5 cycles. A degassed solution of DEA(5.08 g, 27.4 mmol) in methyl alcohol (2 mL) was injected into the flask under a nitrogen atmosphere. Ethyl α-bromoisobutyrate (0.033 g, 0.17 mmol) was injected into the flask *via* a microsyringe. The solution was stirred at 30 ˚Cfor 4 h. The polymerization was stopped by opening the flask and diluting with 100 mL THF. The mixture was passed through an Al_2O_3 column with THF as eluent to remove the catalyst. The eluent was concentrated and poured in cold n-hexane. The resulting PDEA was isolated and then dried in a vacuum (2.7g). ^{1}H NMR spectrum (400 MHz, $CDCl_3$) δ in ppm: 3.9-4.1 (2H), 2.6-2.8 (2H), 2.5-2.6 (4H), 1.7-2.1 (2H), 0.8-1.1 (9H). The number-averaged molecular weight (Mn) of the polymer was measured by GPC to be 2.1×10^4 with a polydispersity of 1.2(**Figure S1b**).

Synthesis of poly [2- (*N*-oxide-*N*, *N*-diethylamino) ethyl methacrylate] (OPDEA) by oxidation of PDEA and fluorescence labelling

PDEA (0.5 g) was dissolved in 4 mL 30% hydrogen peroxide (H_2O_2) solution. The mixture was stirred at room temperature for 4 h and then dialyzed in DI water to completely remove the unreacted H_2O_2. The OPDEA (0.498 g, 92% yield) was obtained after freeze-drying. ^{1}H NMR spectrum (400 MHz, D_2O) δ in ppm: 4.1-4.5 (2 H), 3.1-3.6 (6 H), 1.7-2.1 (2 H), 0.7-1.4 (9H). DEA was copolymerized as described above with 5 mol% 2-[*N*-(*tert*-butoxycarbonyl) amino]ethyl methacrylamide (Boc-AEMA, synthesized according to the reported method)[57] and the resulting copolymer was oxidized using the aforementioned reaction conditions. The copolymer OPDEA/Boc-AEMA (200 mg) was dissolved in DCM (2 mL) and cooled in an ice-bath. TFA (2 mL) was dropped into the solution with stirring for 2 h. The deprotected copolymer was isolated after evaporation of the solvent and TFA. It was then dissolved in PBS (pH 8.0, 2 mL) and with 20 μL DMSO solution of Cy5.5-NHS (10 mg/mL). The solution was

stirred at room temperature for 6 h and then loaded in a dialysis bag (MWCO 3.5 kDa) against deionized water for 48 h, changing the water every four hours. Lyophilization of the solution gave Cy5.5-labeled OPDEA ($^{Cy5.5}$OPDEA) and its fluorescence was confirmed.

Cell culture

The mouse embryonic fibroblasts cells (NIH-3T3), human liver carcinoma cells (HepG2), cervix cancer cells (HeLa), lung carcinoma cells (A549), breast cancer cells (MDA-MB-231) and human breast adenocarcinoma cell line (MCF-7) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HepG2 cells were incubated in DMEM (Life Technology Co., Ltd., Carlsbad, USA) supplemented with 10% heat-inactivated FBS (fetal bovine serum, Life Technology Co., Ltd.), penicillin (100unit mL^{-1}, Genom Biological Technology Co., Ltd., Hangzhou, China) and streptomycin (100 μg mL^{-1}, Genom Biological Technology Co., Ltd.) in a humidified atmosphere of 5% CO_2 at 37^{0}C. The other cells were maintained in RPMI 1640 (Life Technology Co., Ltd.) supplemented with 10% FBS, penicillin (100unit mL^{-1}) and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2 at 37 ^{0}C.

Cell uptake of $^{Cy5.5}$OPDEA and $^{Cy5.5}$PEG

HepG2 cells were seeded in 6-well plates at a density of 2×10^5 cells per well in 2 mL DMEM medium and incubated overnight. $^{Cy5.5}$OPDEA (20μg/mL) was added to each well and incubated with the cells for timed intervals: 30 min, 1 h, 2 h,4 h, 6 h. HepG2 cells were cultured with $^{Cy5.5}$PEG at the same Cy5.5-equivalent dose in 6-well plates as the control. At interval time, the medium was removed and the cells were rinsed with PBS for confocal imaging, or trypsinized, washed twice with PBS and resuspended in PBS for analysis using flow cytometry to obtain the Cy5.5-positive cell percentage (10,000 cells were counted per treatment).

Effects of endocytosis inhibitors on cellular uptake of $^{Cy5.5}$OPDEA

HepG2 cells were seeded in 6-well plates at a density of 2×10^5 cells per well in 2 mL DMEM medium and incubated overnight. The medium was replaced with 2 mL of fresh medium.

Chlorpromazine (an inhibitor of clathrin-mediated endocytosis), filipin III (an inhibitor of caveolae-mediated endocytosis), wortmannin (an inhibitor of phosphatidylinositol 3-kinases-mediated macropinocytosis) or cytochalasin D (an inhibitor of actin-polymerization) was separately added to the medium at the concentrations of 50, 7.5, 5 or 5 μM. After 30 min incubation, $^{Cy5.5}$OPDEA polymer (20 μg/mL) was added to each well and incubated with the cells for 2 h. The medium was removed and the cells were rinsed with PBS, trypsinized, washed twice with PBS and resuspended in PBS, and analysed immediately using flow cytometry to obtain the Cy5.5-positive cell percentage (10,000 cells were counted per treatment).

In vitro cytotoxicity assay

The cytotoxicity of OPDEA was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. Briefly, cells were seeded evenly in 96-well plates (4,000–5,000 cells per well) and incubated for 24 h, followed by the addition of OPDEA at set concentrations for 48 h. Subsequently, 20 μL MTT (5 mg/mL) in PBS was added to each well and incubated at 37 ^{0}C for 4 h. Finally, the medium was removed and replace with 100 μL DMSO. The absorbance intensity in each individual well was determined at 562 nm using a Molecular Devices microplate reader. Each concentration was tested in triplicate and in three independent experiments.

Imaging in vitro mitochondrial targeting of OPDEA

Cells (1×10^5) were plated onto a glass-bottom petri dish and cultured for 24 h. The medium was replaced with fresh medium containing Mito-Tracker Green (100 nM in the culture medium). After 15 min, Hoechst 33342 solution was then added to the petri dish. After another 15 min incubation, $^{Cy5.5}$OPDEA was added to the petri dish at a Cy5.5-equivalent concentration of 0.5 μg/mL. The cells were immediately imaged using a confocal laser scanning microscope (CLSM, Nikon A1). The Hoechst 33342 labelled nuclei were excited using a 405 nm laser, observed at the emission wavelengths of 427~475 nm, and expressed as blue. Mito-Tracker Green was excited using a 488 nm laser, observed at 500~550 nm and expressed as green. Cy5.5

was excited using a 640 nm laser, observed at 662~737 nm and expressed as red. The obtained confocal images were further analysed by Meta Morph or Image J software.

The JC-1 assay of mitochondrial membrane potential

HepG2 cells were seeded in a confocal microscope dish and cultured as described above. The cells were further incubated at 37 ^{0}C in a humidified atmosphere containing 5% CO_2 for 24 h. OPDEA solution (20 μL) in DMEM medium at 20 μg/mL was added to the dish. After 30 min of incubation, JC-1 in DMEM medium was added to the cells at 5 μg/mL to stain the mitochondria for 20 min. After removing the medium and washing with PBS, the cells were immediately imaged using confocal microscopy (Nikon A1). The monomeric form of JC-1 was excited using a 488 nm laser, observed at the emission wavelengths of 500~550 nm, and shown in green. The mitochondrial aggregate form was excited using a 543 nm laser, observed at 570~620 nm, and shown in red. The obtained confocal images were further analysed using Image J software. For flow cytometry analysis, MCF-7 cells were incubated in 6-well plates at a density of 2×10^6 cells per well in 2 mL RPMI 1640 medium and incubated overnight. The OPDEA solution (20 μL) in DMEM medium at 20 μg/mL was added to a well. After 2h incubation, the medium was removed and the cells were rinsed with PBS, trypsinized, washed with PBS and added 0.5 mL fresh medium, and incubated with JC-1 (5 μg/mL) for 20 min at 37 ^{0}C in a humidified atmosphere containing 5% CO_2. Thereafter the cells were centrifuged, resuspended in 500 μL of PBS, and analysed immediately on a flow cytometer (BD FACS Calibur). The carbonyl cyanide 3-chlorophenylhydrazone (CCCP)-treated cells and untreated cells were used as positive and negative controls, respectively.

In vivo cancer cell mitochondrial targeting

HepG2 cells (2×10^6 cells per mouse) were inoculated subcutaneously in female BALB/c nude mice. When the tumour volumes reached about 200 mm^3, the mice were *i.v.* administered with 200 μL of $^{Cy5.5}$OPDEA or $^{Cy5.5}$ PEG solution (Cy5.5-eq. 25 μg/mL). The mice were euthanized at 6 h post injection and the tumours were dissected and weighted. Each weighted tumour was

cut into small pieces and then homogenized in 9 × tumour weight of 0.25 mol/L sucrose solution in Tris-HCl buffer (pH 7.4)). Each homogenate (500 μL) was combined with 500 μL of 0.25 mol/L sucrose solution in Tris-HCl buffer and centrifuged at 1,000 g for 10 min at 4 ^{0}C. The obtained supernatant was carefully transferred to another clean and pre-cooled centrifuge tube and then centrifuged at 13,000 g for 15 min at 4 ^{0}C. The pellet was resuspended in 500 μL of 0.34 mol/L sucrose tris solution and further centrifuged under the same conditions. The pellet was obtained and resuspended in 100 μL of 0.34 mol/L sucrose Tris-HCl solution. The fluorescent intensity of the suspension was measured by Molecular Devices microplate reader. Mitochondrial smears were made by applying 20 μL of the suspension solution onto glass slides, and observed by confocal microscopy. Cy5.5 was excited using a 640 nm laser, observed at 662~737 nm and expressed as red.

Supplemental figures

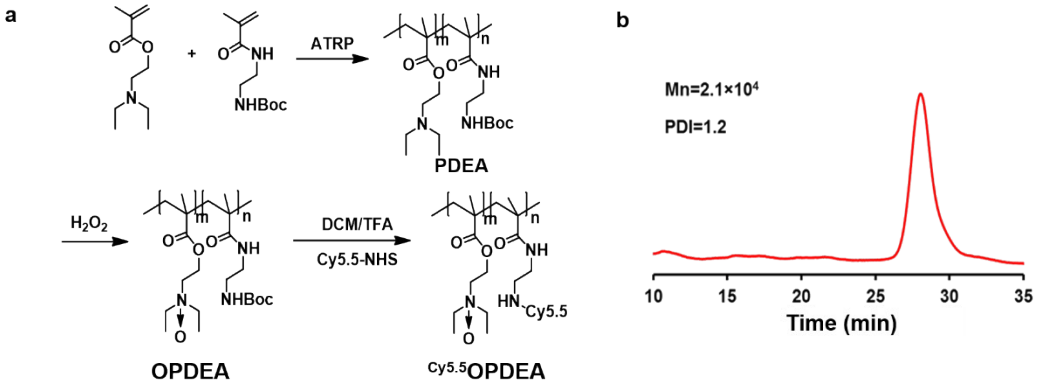

Figure S1. a) The synthesis of the labeled $^{Cy5.5}$OPDEA. b) The GPC trace of PDEA in THF.

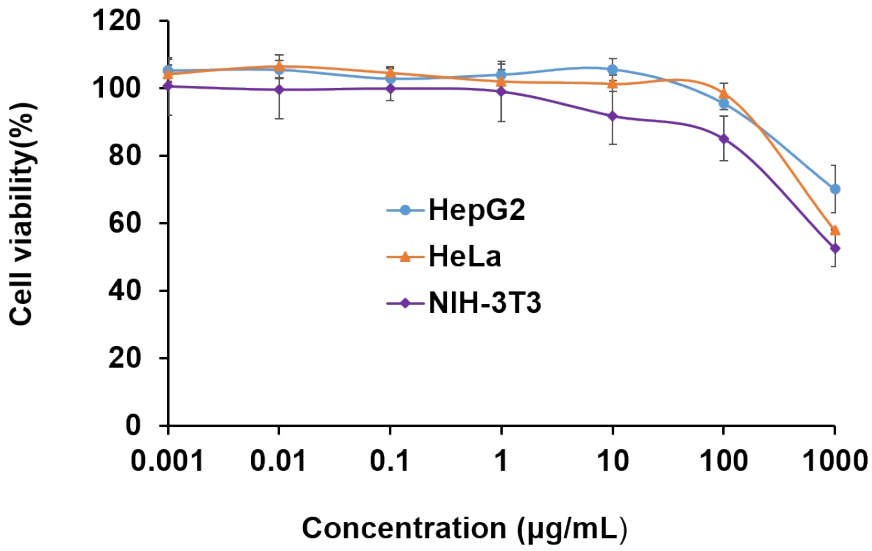

Figure S2. In vitro cytotoxicity of OPDEA against HepG2, HeLa and NIH-3T3 cells.