Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting information

Cancer Cell Selective-killing Polymer/Copper Combination **

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1. Supporting Tables and Figures

Table 1. The IC_{50s} of PDA-PEG/Cu^{2+} nano-complex for normal and cancer cell lines. IC_{50s} were calculated based on MTT results by IBM SPSS Statistics software.

Cell lines	IC_{50} (μM)
MDA-MB-231	2.32±0.33
SKOV-3	3.83±0.58
NCI/ADR-RES	4.72±0.45
UMSCC 22A	5.34±0.51
CT 26	10.56±0.90
HCT 116	11.45±5.35
HL60	22.90±0.83
HH	>831.5
BNL CL.2	552.02±72.50
CONA	289.91±70.00
KC	202.47±26.56
NIH3T3	158.70±17.13
MCF 10A	135.37±10.80

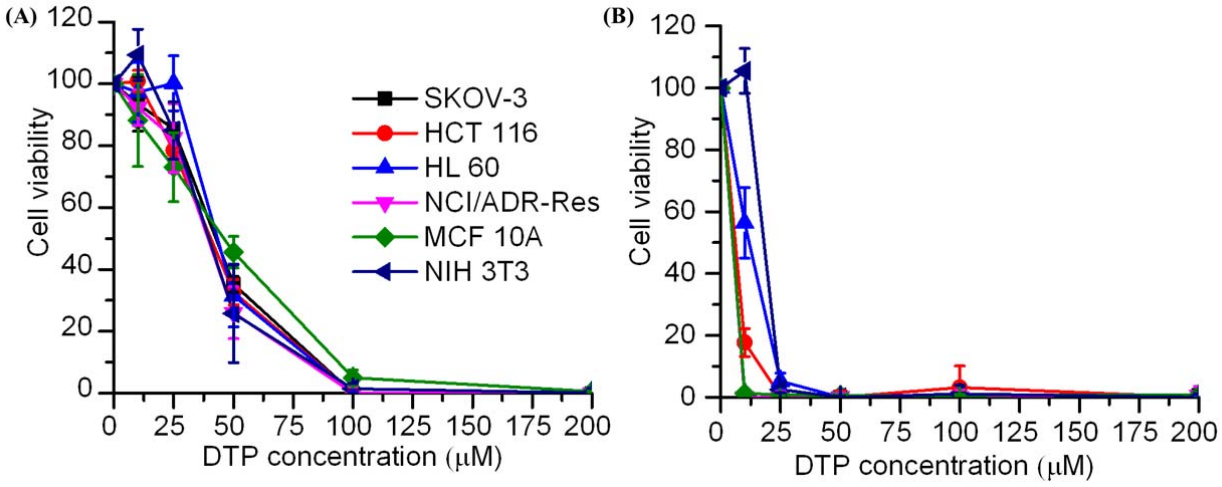

Figure S1. Cytotoxicity of DTP for different cell lines at the absence (A) and presence (B) of 10 μM $CuCl_2$. Data represent the means ± SD, n = 3.

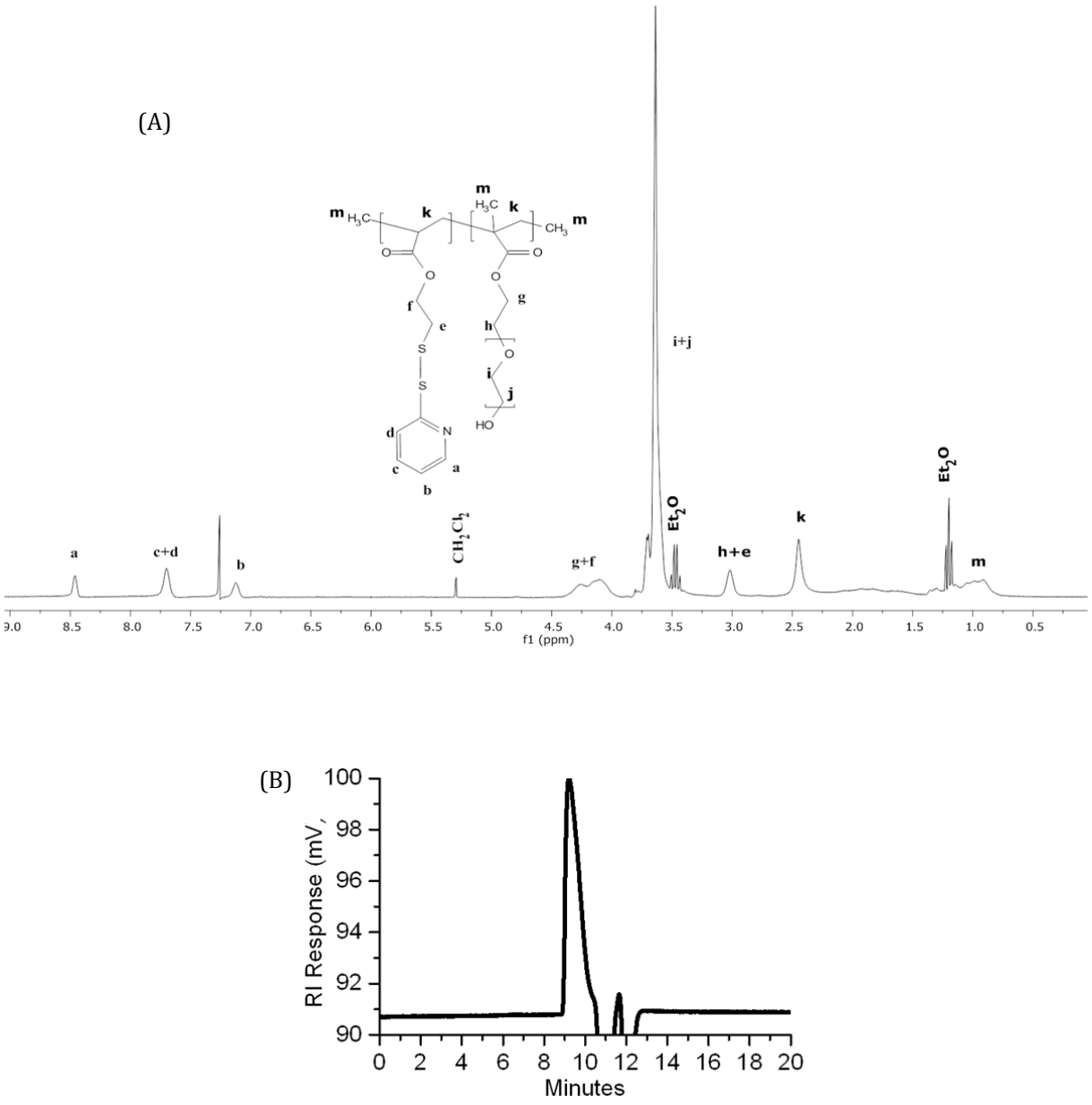

Figure S2. Representative ^{1}H-NMR spectrum (A) and GPC curve (B) for PDA-PEG polymer. The NMR spectrum was acquired in $CDCl_3$.

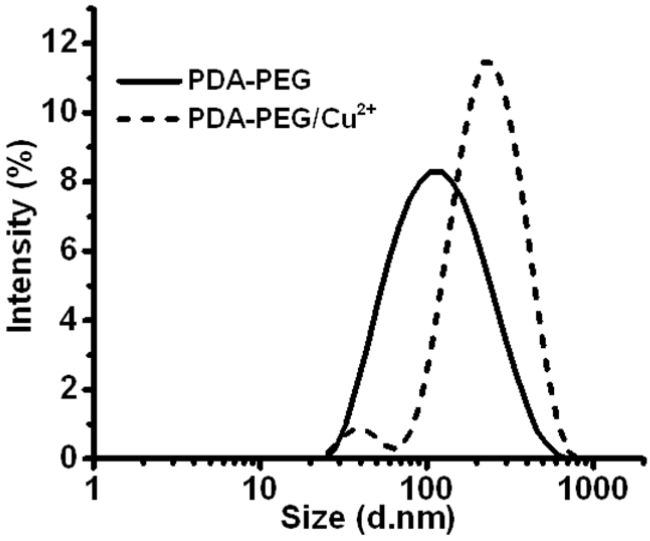

Figure S3. The size distribution of nanoparticles formed from PDA-PEG polymer and PDA-PEG/Cu^{2+} acquired by dynamic light scattering.

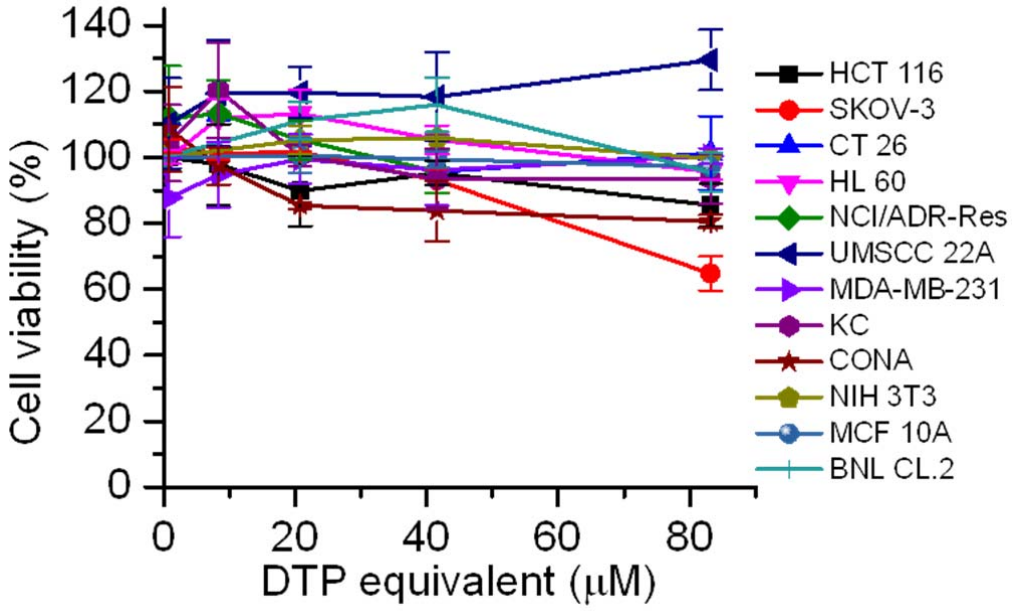

Figure S4. Cytotoxicity of PDA-PEG nanoparticle for different cell lines without the addition of 10 μM $CuCl_2$. Data represent the means ± SD, n = 3.

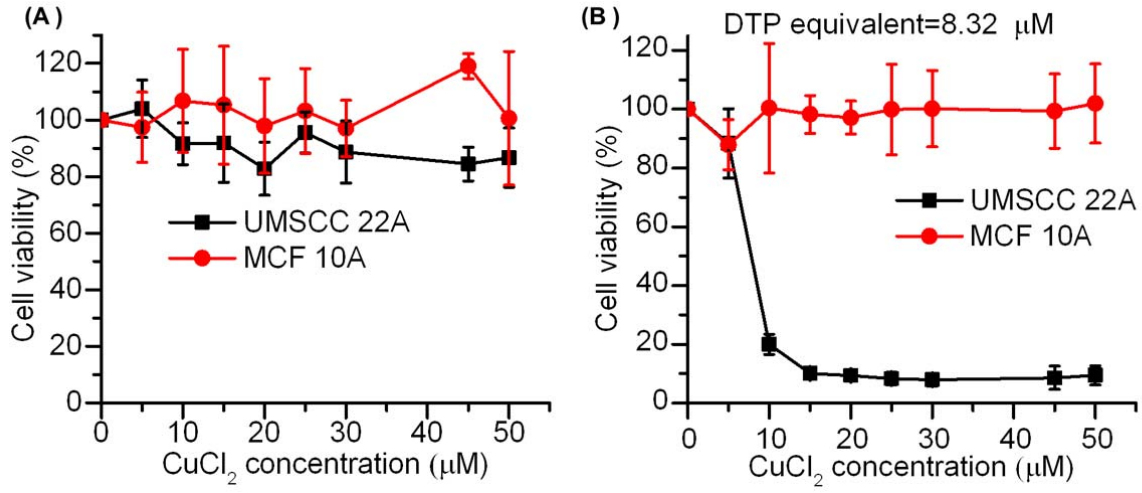

Figure S5. The concentration effect of $CuCl_2$ on the cytotoxicity of medium (A) and PDA-PEG (B). PDA-PEG (8.32 μM, equivalent to DTP) was mixed with different concentrations of $CuCl_2$ and incubated with UMSCC 22A and MCF 10A for 48 h. Data represent the means ± SD, n=3.

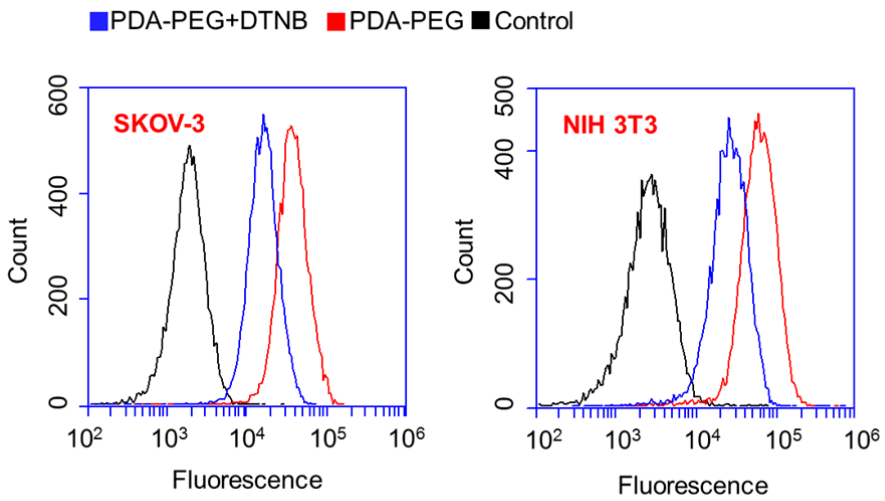

Figure S6. The flow cytometry spectra of SKOV-3 (Left) and NIH 3T3 (Right) cells treated with Cy5 labeled PDA-PEG nanoparticles. Cellular uptake assays were carried out 1 h after the addition of nanoparticles

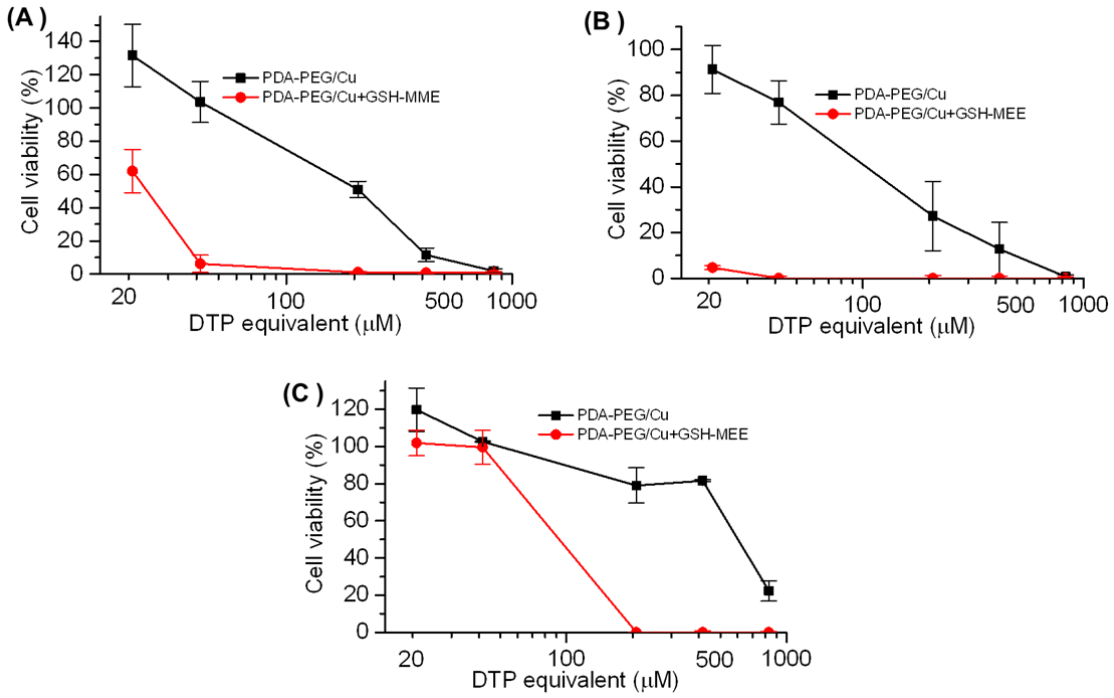

Figure S7. The effect of GSH-MME on the cytotoxicity of PDA-PEG/Cu^{2+} for KC (A), NIH 3T3 (B), and BNL.CL.2 (C) cells. Data represent the means ± SD, n=3.

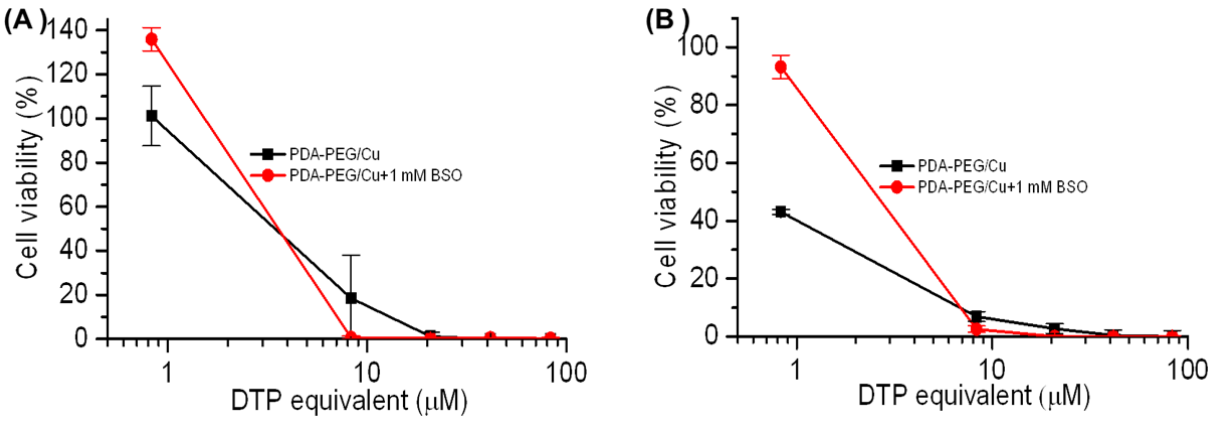

Figure S8. The effect of BSO on the cytotoxicity of PDA-PEG/Cu^{2+} for UMSCC 22A (A) and SKOV-3 cells. Data represent the means ± SD, n=3.

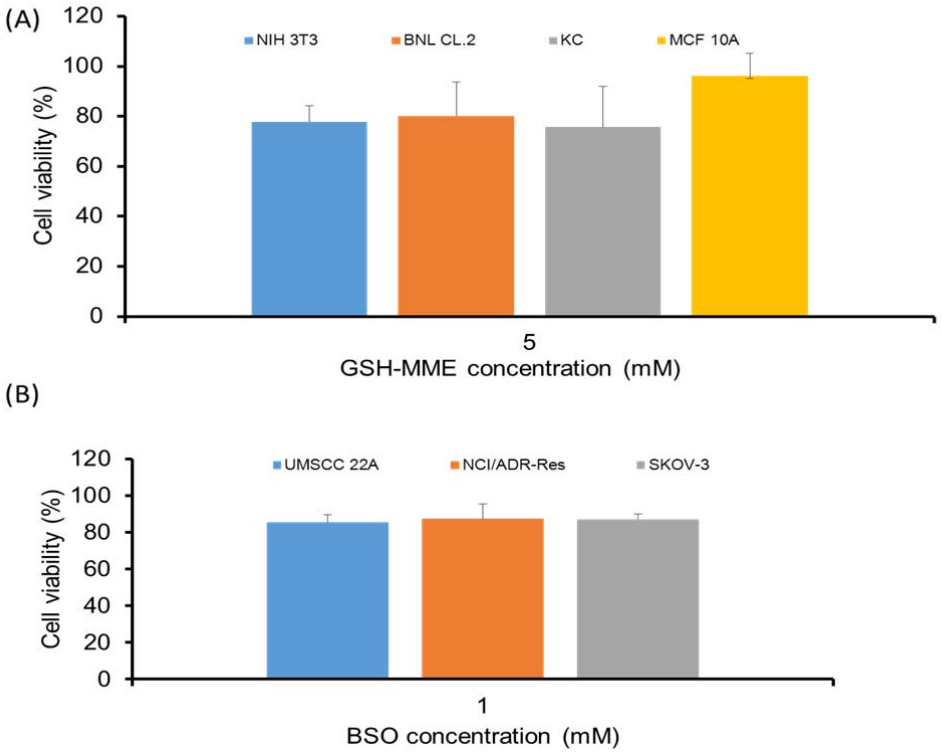

Figure S9. The effect of GSH-MME (A) and BSO (B) on cell viability. Data represent the means ± SD, n=3.

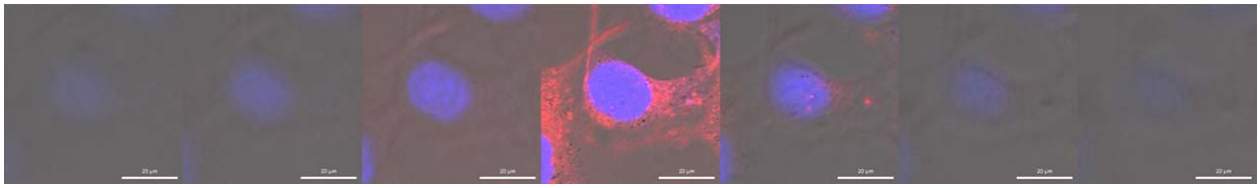

Figure S10. The Z-serial optical sectioning confocal images of SKOV-3 cells after incubating with PDA-PEG nanoparticle for 1 hr. The step of the section was 5.1 μm. Scale bars were 20 μm.

2. Experimental Section

2.1. Materials and Synthesis

2.1.1. Materials: Aldrithiol-2 and Silica gel (Spherical, 100 mm) were purchased from Tokyo Chemical Industry Co., LTD (Harborgate Street, Portland, OR). 2-Mercaptoethenol, DL-dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), 2, 2-Azobisisobutyronitrile (AIBN), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Propidium Iodide (PI) and Poly(ethylene glycol)methacrylate (Mn=360 Da) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Buthionine sulfoximine (BSO) and Glutathione monomethylester (GSH-MME) were purchased from Cayman Chemical (Ann Arbor, Michigan). Penicillin (10,000 U/mL), streptomycin (10,000 mg/mL), 0.25% trypsin-EDTA, Dulbecco's Modified Eagle Medium (with L-glutamine) and fetal bovine serum (FBS) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Molecular probes and RNase were purchased from Life Technologies (Grand Island, NY). GSH-Glo™ Glutathione Assay kit was purchased from Promega Corporation (Madison, WI). Cy5-NHS was purchased from Lumiprobe Corporation (Hallandale Beach, FL). All the other solvents used in this research were purchased from Sigma Aldrich Chemical Co. (St.Louis, MO) and used without further purification unless otherwise noted.

2.1.2. Synthesis of poly[(2-(pyridin-2-yldisulfanyl)ethyl acrylate)-co-[poly(ethylene glycol)]] (PDA-PEG) : PDA-PEG polymer was synthesized according to our previous reports. Briefly, 2-(pyridin-2-yldisulfanyl)ethyl acrylate (PDA, 241.3 mg, 1 mmol) and poly(ethylene glycol) methacrylate (PEG_{360}, Mn=360 Da, 360 mg, 1 mmol) were dissolved in 10 mL degassed anisole. 2,2-Azobisisobutyronitrile (AIBN, 14 mg, 0.085 mmol) in 1 mL degassed anisole was then added, and the reaction mixture was stirred for 24 h at 65 ^{o}C. The final product was precipitated (3×) in ice cold ether and dried for 48 h in vacuum. The structure of

PDA-PEG was confirmed by ^{1}H-NMR, and its molecular weight and polydispersity were evaluated by gel permeation chromatography (GPC).

2.1.3. PDA-PEG-Cy5 synthesis: PDA-PEG was modified by Cy5 for cellular uptake study. Briefly, cysteamine (0.11 mg, 5% PDA function group) in 500 µL DMSO was added dropwise into 20 mg PDA-PEG in 500 µL DSMO and the reaction mixture was kept at room temperature overnight. After overnight reaction, Cy5-NHS ester (0.39 mg in 390 µL DMSO) was added and the mixture was kept for reacting overnight, following a thoroughly dialysis towards DMSO to remove free Cy5. The concentration of Cy5 in the final product was measured by a microplate reader (SpectraMax® M5, Molecular Devices Inc) at λ_{ex}640 and λ_{em}680.

2.2. Characterization

2.2.1. PDA quantification in PDA-PEG polymer: Two methods were used to measure PDA concentration in the polymer. In the first method, PDA-PEG (50 µg/mL in DMSO) was incubated with tris(2-carboxyethyl)phosphine (TCEP, 10 mM, 20 mM and 50 mM) for 1 hour at room temperature, and then the amount of pyridine-2-thione released was quantified through UV-Vis spectrophotometer at λ = 375 nm and correlated to PDA amount (ε, molar absorption coefficient = 8080 $M^{-1}cm^{-1}$). In the second method, a calibration curve of pyridine-2-thione was firstly established and then applied to calculate the PDA concentration in the polymer. In brief, 100 µg aldrithiol-2 was dissolved in 1 mL DMSO and completely converted to pyridine-2-thione by adding excess TCEP (13.1 mg, 100× excess). The reaction mixture was then diluted in DMSO to obtain a serial of concentrations of pyridine-2-thione with UV-Vis absorbance between 0.1 and 1 at 375 nm. Based on the UV-Vis absorbance, the calibration curve was established. PDA-PEG (50 µg/mL in DMSO) was incubated with 10, 20 and 50 mM TCEP for

1 h. After that, PDA concentration in the polymer was calculated according to the calibration curve after measuring the UV-Vis absorbance of the polymer solution.

2.2.2. TEM and DLS: The size and morphology of PDA-PEG in PBS is characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS). In brief, 1 mg PDA-PEG was dissolved in 1 mL PBS with or without copper chloride ($CuCl_2$, 10 μM). Then the PDA-PEG solution was measurement by DLS (Zetasizer Nano ZS, Malvern Instruments Ltd), or loaded onto carbon coated copper grids, dried and observed directly under TEM (Hitachi H8000, Hitachi High Technologies America, Inc.).

2.2.3. PDA-PEG Stability Study In Vitro: The stability of PDA-PEG was investigated at the presence of varied concentrations of GSH to mimic the conditions in the body. Briefly, 50 μg PDA-PEG was dissolved in 1 mL 10 mM phosphate buffer saline with 10 μM $CuCl_2$ and varied concentrations of GSH (0, 0.01, 0.1, 1 and 10 mM). The UV-Vis absorbance of the polymer at 0, 0.5, 1, 1.5 and 2 h after adding GSH was measured by a UV-Vis spectrophotometer and normalized by comparing to the maximum absorbance of the polymer.

2.3 Intracellular GSH measurement

Tumor and normal cells were seeded in 96-well white plate (5,000 cells/well) overnight prior to the study. BSO (1 mM) or GSH-MME (5 mM) in culture medium was added. For control group, only fresh culture medium was added. Cells were incubated for 8 h, and the intracellular GSH concentration was measured by GSH-Glo™ Glutathione Assay according to the manufacturer's instruction.

2.4. In Vitro Cytotoxicity Assay

2.4.1 MTT Assay: In vitro cytotoxicity of PDA-PEG was tested in cancer cell lines (MDA-MB-231, SKOV-3, NCI/ADR-Res, UMSCC 22A, HCT 116 and HL 60) and normal cell lines (CONA, NIH 3T3, MCF 10A, KC and BNL CL.2). Cells were seeded in 96-well plate (20,000 cells/well) for 24 h prior to the study. Then a serial of concentrations of PDA-PEG in culture medium was added, supplementing with or without $CuCl_2$ (10 μM). The cells were then incubated 48 h in in 95/5% air/CO_2 at 37 ^oC. After 48 h, MTT reagent (100 μL, 10%(w/w) in medium) was added and incubated for 4 h, following the addition of MTT stop solution and the measurement of the optical density of the medium using a microplate reader (ELX808, Bio-Tech Instrument, Inc) at λ = 595 nm.

2.4.2 Intracellular GSH effect to cytotoxicity: To test the effect of intracellular GSH effect to the cytotoxicity of PDA-PEG, intracellular GSH concentration was inhibited or promoted by BSO and GSH-MEE, respectively. In brief, cells were seeded in 96-well plate (20,000 cells/well) for 24 h prior to the study. To investigate the PDA-PEG cytotoxicity responding to reduced intracellular GSH, cells was treated with BSO (1 mM) for 24 h and then varied concentrations of PDA-PEG with or without 10 μM $CuCl_2$ were added and incubated for another 48 h. To investigate the PDA-PEG cytotoxicity responding to increased intracellular GSH, cells were treated at the same time with GSH-MME (5 mM) and varied concentration of PDA-PEG with or without 10 μM $CuCl_2$ for 48 h. For both experiments, the cell viability was finally quantified by MTT assay.

2.5. Cellular Uptake Study

2.5.1. Confocal microscopy: SKOV-3 cells (200,000 cells/dish) were seeded in 35mm^2 Petri dishes (Mat Tek, MA, USA) overnight. Prior to adding polymer, certain dishes were added with

5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.6 mg in 75 µL PBS) to block the thiol groups on the cell surface. Then PDA-PEG-Cy5 in 5 µL DMSO was added (equivalent to 0.1 µg/mL Cy5). After 1 h incubation under a humidified atmosphere of 95/5% air/CO_2, cells were washed by PBS (3×), fixed with formaldehyde (4.5 % in PBS) and stained with Hoechst 33342 (final concentration 1 µg/mL). Then cells were analyzed under a confocal microscope (LSM 700, Carl-Zeiss Inc.).

2.5.2. Flow cytometry: SKOV-3 and NIH 3T3 cells (300,000 cells/well) were seeded in 6-well plates overnight. Certain wells were then added with DTNB (0.6 mg in 75 µL PBS) to block the thiol groups on the cell surface, following the addition of PDA-PEG-Cy5 in 5 µL DMSO (equivalent to 1 µg/mL Cy5). After 1 h, cells were washed, trypsinized and resuspened in PBS. Cy5 positive cell population was quantified at λ_{ex} 640 nm and λ_{em} 675 nm using flow cytometry (BD Accuri C6, BD Biosciences).

2.6. Fluorescent Microscopy for Cancer Cell Selective Cell Killing Assay

NIH 3T3, NCI/ADR-Res, SKOV-3, and UMSCC 22A cells were trypsinized and washed by PBS (3×) and resuspended in 1 mL PBS supplemented with Cell Tracker™ deep red dye (2 µM), blue dye (50 µM), green CMFDA dye (25 µM) and orange CMTMR dye (25 µM), respectively. All cells were stained for 30 min at 37 ^{o}C and washed by PBS (3×) and resuspended in 2 mL DMEM culture medium. Cells were then counted and seeded in 24-well plate separately or together. Four wells for each individual cell line and cell mixture. Cell density was 10,000 cells/well for individual cell and 40,000 cells/well for cell mixture. All cells were incubated for 24 h under a humidified atmosphere of 95/5% air/CO_2 before polymer treatment. After 24 h, the culture medium was removed and replaced with fresh medium containing different concentrations of PDA-PEG (DTP equivalent to 8.32-20.79 µM PDA) at

the presence of 10 μM $CuCl_2$. After 24 h of incubation, cells were directly observed under the fluorescence microscopy (Olympus IX81, Olympus America Inc).

2.7. Microarray Assay

2.7.1. RNA isolation: SKOV-3, NCI/ADR-Res, and MCF 10A (5,000,000 cells/well) were seeded in 100 mm Petri dishes (four dishes for each cell line) overnight under a humidified atmosphere of 95/5% air/CO_2. Culture medium was then replaced with fresh one with or without PDA-PEG (41.58 μM DTP) at the presence of 10 μM $CuCl_2$. After that, cells were incubated for another 12 h. Total RNA for gene expression analysis was isolated from cell line samples using miRNeasy Mini Kit (QIAGEN, Cat#. 217004) according to the manufacturer's instructions. Briefly, cells were scraped with 700 μL of QIAzol reagent, collected in eppendorf tube, lysed by vortexing and homogenized by centrifugation through QIAshredder columns (QIAGEN, Cat#. 79656). After addition of 140 μL of chloroform, the homogenate was vigorously shaken for 15 s and centrifuged at 12,000 g for 15 min at 4°C. The RNA-containing aqueous phase was transferred to a new tube and RNA was precipitated with 525 μL of 100% ethanol. Subsequently, the sample was transfer to a RNeasy Mini spin column and centrifuged at 12,000 g for 15 s at room temperature. In the next step, RNA samples were on-column DNase treated and posteriorly washed with RPE buffer. Then, RNA was eluted from the column with 30 μL of RNase free water and quantified using a spectrophotometer. RNA quantity was assessed using an Agilent 2100 Bioanalyzer and RNA Integrity Numbers (RIN) ranged from 9.2 to 10.0.

2.7.2. mRNA labeling and hybridization: Microarrays experiments were performed using Agilent's platform. Total RNA samples were amplified and labeled using Agilent's Low Input Quick Amp Labeling Kit (Cat. # 5190-2306) according to the manufacturer recommendations.

Briefly, mRNA contained in 200 ng of total RNA was converted into cDNA using a poly-dT primer that also contains the T7 RNA polymerase promoter sequence. Subsequently, T7 RNA polymerase was added to cDNA samples to amplify original mRNA molecules and to simultaneously incorporate cyanine 3- or cyanine 5-labeled CTP (cRNA) into the amplification product. In addition, Agilent RNA spike-in controls (Cat.# 5188-5279) were added to samples prior cDNA synthesis and were used as experimental quality control. In the next step, labeled RNA molecules were purified using Qiagen's RNeasy Mini Kit (Cat.# 74104). After spectrophotometric assessment of dye incorporation and cRNA yield, samples were store at -80 °C until hybridization. Labeled cRNA samples were hybridized to SurePrint G3 Human Gene Expression 8x60K v2 Microarrays (Cat.# G4858A-039494) at 65 °C for 17 h using Agilent's Gene Expression Hybridization Kit (Cat.# 5188-5242) according to the manufacturer's recommendations. Two (2) control sample replicates we hybridized against two (2) polymer treated sample replicates in a dye swap design. After washes, arrays were scanned using an Agilent DNA Microarray Scanner System (Cat.# G2565CA).

2.7.3. Data analysis: Data was extracted from images with Feature Extractor Software version 10.7.3.1 (Agilent). In this process, background correction using additive and multiplicative detrending algorithms was performed. In addition, linear and LOWESS methods were used for dye normalization. Subsequently, data was uploaded into GeneSpring GX version 13.0 for analysis. In this process, data was log2 transformed, quantile normalized and base line transformed using the median of all samples. Then, data was filtered by flags in a way that 100% of the samples in at least one of the two treatment groups have a "detected" flag. Differentially expressed genes were determined using a moderated t-test statistics setting as cutoff values a p-value of 0.001 and a fold of 3.0.

2.8. Cell Cycle Analysis

SKOV-3 cells (300,000 cells/well) were seeded in 6-well plates overnight. The culture medium was then replaced by fresh one with PDA-PEG polymer (equivalent to 41.58 μM DTP) at the presence or absence of 10 μM $CuCl_2$. The cell was cultured for 12 h under a humidified atmosphere of 95/5% air/CO_2. Then cells in each well were collected after trypsinization. Cells were wash with PBS (1×) and fixed by 70 % ice cold methanol for 5 min. After the fixation, cells were wash by PBS (1×) and resuspended in 250 μL RNase (100 μg/mL in PBS) for 15 min at 37 ^{o}C. Cells were wash by PBS (1×) again and stained by propidium iodide (PI, 100 μg/mL in PBS) for 1 h on ice. Cells were finally washed and measured by flow cytometry (Ex 488 nm, Em 585 nm).